

Genetic structure of natural populations of *Plasmodium falciparum*

Hamza Ali Babiker

Submitted to the University of Edinburgh for the degree of Doctor of Philosophy

Institute of Cell, Animal & Population Biology, University of Edinburgh

March 1994



I dedicate this thesis with love to my family.

Table of contents	Page number
Dedication	11
Acknowledgements	V111
Abstract	1X
List of tables	X1
List of Figures	X111
List of Abbreviations	XV
Chapter 1. Introduction	1
1.1. Purpose of the study	2
1.2. Malaria: global view	2
1.3. The malaria parasite	3
1.3.1. Life cycle	3
1.3.2. Cytogenetics and organisation of the <i>Plasmodium</i> genome	7
1.3.3. Genetics	9
1.4. Malaria control strategies	10
1.4.1. Malaria control with the vector as target	10
1.4.2. The role of chemotherapy	11
1.4.3. Malaria vaccines	13
1.5. Genetic diversity of the malaria parasite	14
1.6. Polymorphic genetic characters used in this study	15
1.6.1. Enzymes	15
1.6.2. Proteins detected by 2D-PAGE	16
1.6.3. Antigens	16
1.6.3.a. Merozoite surface protein 1, MSP-1	16
1.6.3.b. Merozoite surface protein 2, MSP-2	21
1.6.3.c. Other polymorphic antigens	24
1.6.4. Variation in drug susceptibility	25
1.7. Genetic structure of natural parasite populations	26
1.7.1. Hypothesis of panmictic structure of <i>P. falciparum</i>	29
1.7.2. Hypothesis of clonal structure of <i>P. falciparum</i> .	30
1.7.3. Implication of the genetic structure on the epidemiology and control of malaria	31
1.8. Aim of the thesis	32

Chapter 2. Material and Methods

2.1.	Study areas	33
2.1.1.	Asar village, eastern Sudan	33
2.1.2.	Khartoum, central Sudan	
2.1.3.	Michenga village, south-east Tanzania	35
2.2.	Collection of samples of <i>P. falciparum</i>	37
2.2.1.	Diagnosis of <i>P. falciparum</i>	37
2.2.2.	Collection of blood samples	37
2.2.3.	Collection of mosquitoes	37
2.3.	Preservation and <i>in vitro</i> cultivation of <i>P. falciparum</i>	38
2.3.1	Freezing and storage of <i>P. falciparum</i> isolates	38
2.3.2	Thawing of deepfrozen <i>P. falciparum</i> isolates	39
2.2.3	RPMI culture medium	39
2.3.4	<i>In vitro</i> culture of <i>P. falciparum</i> isolates	40
2.4	Cloning of <i>P. falciparum</i>	40
2.5.	Characterization of <i>P. falciparum</i> isolates for enzymes, proteins, drug response and antigens	41
2.5.1	Enzyme electrophoresis	41
2.5.1.1	Preparation of parasite material	41
2.5.1.2	Cellulose acetate electrophoresis (CAE)	42
2.5.2.	Two dimensional polyacrylamide gel electrophoresis (2DPAGE)	45
2.5.2.1	Incorporation of radio-labelled methionine into parasites	45
2.5.2.2	Preparation of parasites for electrophoresis	45
2.5.2.3	2D gel electrophoresis	46
	(a) First dimension gel	46
	(b) Second dimension gel	46
2.5.2.4.	Fluorography	47
2.5.2.5.	Identification of polymorphic proteins	47
2.5.3.	Susceptibility of <i>P. falciparum</i> to chloroquine, pyrimethamine and mefloquine	50
2.5.3.1.	Set-up of microtiter plates	50
2.5.3.2.	Drug concentrations	50
2.5.3.3.	Interpretation of the test	51
2.5.4.	Characterization of three polymorphic antigens using monoclonal antibodies	51
2.5.4.1.	Preparation of antigen slides	51
2.5.4.2.	Monoclonal antibodies (Mabs)	52
2.5.4.3.	Indirect immunofluorescence assay (IFA)	52
2.5.4.4.	Identification of serotypes of MSP-1, MSP-2 & Exp-1 antigen	53
2.6.	Typing of two polymorphic antigen genes by Polymerase Chain Reaction (PCR) and hybridization with allele-specific oligonucleotides probes	57
2.6.1.	Preparation of DNA from parasite cultures	57

2.6.2. Isolation of DNA from fingerprick blood samples	57
2.6.3. Extraction of DNA from oocysts and from parasites acquired by fed mosquitoes	58
2.6.4. Polymerase Chain Reaction (PCR)	59
2.6.4.1. PCR conditions	60
2.6.4.2. Agarose electrophoresis	60
2.6.5. Southern blotting of PCR-amplified fragments	61
2.6.6. Labelling and detection of allele specific oligonucleotide probes by the enhanced chemiluminescence (ECL) system	62
2.6.6.1. Sequence-specific oligonucleotide probes	62
2.6.6.2. Labelling of oligonucleotide probes	63
2.6.6.3. Hybridization and detection of the probes	63
2.6.6.4. Stripping of the probes	64
Chapter 3: Genetic diversity of <i>Plasmodium falciparum</i> in Sudan	65
3.1. Summary	65
3.2. Subjects and methods used	65
3.3. Results	66
3.4.1. Diversity of <i>P. falciparum</i> isolates in Asar	66
3.4.2. Diversity of isolates collected from the same household in Asar	72
3.4.3. Diversity of <i>P. falciparum</i> isolates collected in Khartoum	73
3.4.4. Comparison between isolates in Khartoum area and Asar	74
3.4. Discussion	80
Chapter 4: Seasonal variations in the <i>Plasmodium falciparum</i> population in Asar village	84
4.1. Summary	84
4.2. Subjects and methods used	84
4.3. Results	85
4.3.1 Variants of enzymes, 2D-proteins, antigens over two consecutive transmission seasons, 1989 and 1990	85
4.4.3. Comparison of alleles of polymorphic regions of MSP-1 and MSP-2 genes, between 1989-1991	91
4.4 Discussion	102
Chapter 5: Diversity of drug responses in <i>Plasmodium falciparum</i> in Asar village, Sudan	106
5.1. Summary	106
5.2. Introduction	106
5.3. Methods used	107
5.4. Results	108
5.4.1. Responses to chloroquine, pyrimethamine & mefloquine of 1989 isolates and clones	108
5.4.2. Comparison of responses to chloroquine & pyrimethamine, in 1989 and 1990	111
5.4.3. Genetic evidence for R1 type chloroquine resistant <i>P. falciparum</i>	112
5.4.3.1. <i>In-vivo</i> tests	113
5.4.3.2. <i>In-vitro</i> chloroquine response of the isolates which caused the primary infections	113
5.4.3.3. MSP-1 and MSP-2 of primary and recrudescence parasites	113
5.5. Discussion	117

Chapter 6: Population genetic structure of <i>Plasmodium falciparum</i> in Michenga village, Tanzania: frequency of crossing in wild-caught mosquitoes	120
6.1. Summary	120
6.2. Methods used	120
6.3. Results	123
6.3.1. Allelic polymorphism of the MSP-1 & the MSP-2 in blood forms	123
6.3.2. Multiclonal infection	125
6.3.3. Genotype diversity of oocysts infecting wild mosquitoes	128
6.3.4. Frequency of hybrid oocysts	133
6.3.5. Population genetic analysis	138
6.4. Discussion	141
Chapter 7: Dynamics and polymorphism of <i>Plasmodium falciparum</i> parasites at the household level in Michenga village, Tanzania	145
7.1. Summary	145
7.2. Methods used	145
7.3. Results	146
7.3.1. Genotype of oocysts infecting mosquitoes in different households	146
7.3.2. Variations in allele frequencies of MSP-1 & MSP-2 within different houses	149
7.3.3. Parasites infecting individuals in each household	150
7.3.4. Comparison of parasites infecting people, parasites acquired by fed mosquitoes, and oocysts of a single hut (8001/1)	151
7.4. Discussion	156
Chapter 8: Comparison of <i>Plasmodium falciparum</i> populations in Asar village, Sudan with Michenga village, Tanzania	159
8.1. Summary	159
8.2. Subjects and Methods	159
8.3. Results	160
8.3.1. Comparison of parasite rate and entomological inoculation rate (EIR) between Michenga and Asar	160
8.3.2. Polymorphism of MSP-1 and MSP-2 in Michenga	161
8.4.3. Polymorphism of MSP-1 and MSP-2 in Asar	161
8.4.4. Comparison of MSP-1 and MSP-2 alleles in Michenga and Asar	166
8.4. Discussion	169
Chapter 9: General discussion and conclusions	173
References	184
Appendix 1. Summary of the model used for population genetic analysis	198
Appendix 2. Publications from this work	200

Acknowledgements

I am extremely grateful to my supervisor Professor David Walliker for his continuing support and encouragement during the course of this work.

I thank the staff of Gedaref Hospital, the malaria committee of Gedaref town and the villagers of Asar, Sudan, without their support and cooperation this work would not have been possible. I acknowledge the continuing support of the Malaria Administration of the Ministry of Health, Sudan, Dr. Gweria Satti, the director of the Sudan malaria project and the international collaborators with whom I shared some facilities for sample collections in Sudan. I would like to thank my colleague Dr. Ali Sultan who provided medical service and helps during the field work in Sudan.

I am grateful to Professor W. Kilama, Director-General, NIMR, Tanzania, and to Professor Marcel Tanner, Swiss Tropical Institute, Basel, for their support. Dr. Dereck Charlwood and the entomology team of Ifakara Centre, Medical Research Council Tanzania, provided technical help in collection of mosquitoes and blood samples from Michenga village. Dr Thomas Teucher and the research team in Ifakara centre provided help and support. I am grateful to the excellent co-operation of the villagers in Michenga village, Tanzania.

Advice on statistical analysis was kindly received from Mrs Dorothy Currie. Dr. Lisa Ranford-Cartwright and Miss Alison Creasey has given an appreciated technical help.

I am grateful to the British Council, for providing me with a research studentship.

Many people contributed advice and helpful discussion during the course of this work, especially Professor Geoffrey Beale, Dr. Jana McBride, Dr. Richard Carter, Dr. David Arnot, Professor W. H. Hill, Dr. Andrew Read and other colleagues in this department.

Thanks to Mr. Frank Johnston and Mr. Graham Brown for their photographic services.

My sincere gratitude to my wife and my son who shared the burden of this work and provided moral support and encouragement.

Abstract

Genetic polymorphism of *Plasmodium falciparum* populations has been studied in two localities in Sudan, Khartoum the capital and Asar village in the eastern region. The frequencies of alleles of three antigens denoted MSP-1, MSP-2 & Exp-1, three enzymes and nine polymorphic proteins detected by 2D-PAGE were investigated. Parasites from both areas were found to be very diverse, and frequently patients were infected with more than one genotype. Allelic polymorphism was slightly lower in Asar village than in Khartoum.

In Asar village, the antigens, 2D-PAGE proteins and enzymes, were again monitored in 1990. In addition, alleles of MSP-1 and MSP-2 were characterised by PCR-amplification and allele-specific DNA probes in, 1989, 1990 and 1991. Some changes in the frequencies of certain alleles were noted from one year to another.

In vitro responses to chloroquine, pyrimethamine and mefloquine, were assessed among the parasites of Asar in 1989 and 1990. The parasites were found to have diverse responses to chloroquine and pyrimethamine in both years. The prevalence of parasites sensitive and resistant to pyrimethamine varied considerably between 1989 and 1990, but the responses to chloroquine remained more or less stable. Characterization of R1-type chloroquine resistant parasites for antigen genes proved that they were genuine recrudescant resistant parasites, and not derived from new infections.

The breeding structure of *P. falciparum* in Michenga was analysed by examining the extent of crossing between parasite clones in wild-caught mosquitoes *Anopheles gambiae* and *A. funestus* were genotyped. Individual oocysts in the mosquitoes were typed for alleles of MSP-1 & MSP-2 by PCR and allele-specific probes. 16 alleles of the MSP-1 and 24 of the MSP-2 were identified within 71 oocysts collected within two week period. The majority of oocysts were heterozygous, containing two different alleles of either MSP-1 and/or MSP-2. The observed frequencies of oocyst

genotypes were in accordance with random mating occurring between the parasites in the community.

Allele frequencies of the MSP-1 and MSP-2 gene of individual oocysts collected simultaneously in four households widely separated in Michenga village were compared. There was no evidence of genotype clustering within these households.

The MSP-1 & MSP-2 alleles identified by the PCR/DNA probes and frequency of multiclonal infections in Asar in 1991 were compared to those of parasites collected in the same year in a village of similar size, Michenga, in Tanzania. Malaria in Michenga is holo-endemic and transmission is perennial; in contrast in Asar, Sudan, malaria is meso-endemic and transmission is limited to 12-14 weeks. In Michenga, the numbers of alleles of the two genes were much greater than in Asar. In addition a higher frequency of multiclonal infections was detected in Michenga.

List of tables

Table 1. Electrophoretic conditions and staining solutions for enzymes, GPI, ADA and PEP.

Table 2. Monoclonal antibodies used to characterise serotypes of MSP-1, MSP-2 & Exp-1 antigens.

Table 3. PCR primer used to amplify a region of variable number of tandem repeats (VNTR) on MSP-1 & MSP-2.

Table 4. Allele-specific oligonucleotide probes of MSP-1, & MSP-2.

Table 5. Detailed characterisation of 29 isolates from Asar collected in 2 weeks period in 1989.

Table 6. Association between alleles of proteins L, N and F in isolates of *P.falciparum* from Asar.

Table 7. Characteristics of *P.falciparum* of two household pairs in Asar

Table 8. Characterisation of 23 *P.falciparum* isolates from Khartoum collected in 1988.

Table 9. Detailed characterisation of 16 *P.falciparum* isolates from Asar collected in 1990.

Table 10. Comparison of allele frequencies of MSP-1 gene between 1989 and 1991 in Asar village.

Table 11. Comparison of frequencies of K1 allele between 1989 and 1991 in Asar village

Table 12. Comparison of frequencies of MAD20 allele between 1989 and 1991 in Asar village

Table 13. Comparison of frequencies of RO33 allele between 1989 and 1991 in Asar village

Table 14. Comparison of allele frequencies of MSP-2 between 1989 and 1991 in Asar village.

Table 15. *In-vitro* drug responses of 28 *P. falciparum* isolates from Asar village, 1989.

Table 16. *In-vitro* drug responses of 20 *P. falciparum* clones from Asar village, 1989.

Table 17. Comparison of chloroquine and pyrimethamine susceptibility of *P. falciparum* isolates from Asar village, during 1989 and 1990.

Table 18. *In vitro* chloroquine responses of parasites of initial infections from six patients suspected to have R1 type of chloroquine resistance, taken before commencement of drug-treatment.

Table 19. Alleles of MSP-1 and MSP-2 genes of primary and recrudescent (R1) *P. falciparum* in patients treated with chloroquine.

Table 20. Observed combinations of alleles based on sequence of MSP-1 & MSP-2 genes within isolates collected from Michenga on 21st, 24th & 25th June 1991.

Table 21. The minimum and maximum number of clones per isolate within 53 isolates collected from the village.

Table 22. Observed two-locus genotypes of 60 oocysts.

Table 23. Analysis of 77 oocysts collected from wild-caught mosquitoes of Michenga village.

Table 24. Comparison of observed and expected number of homozygotes and heterozygotes assuming Hardy Weinberg predictions. X

Table 25. Observed homozygosity at each locus and two-locus homo- and heterozygosity. X

Table 26. Analysis of oocyst data with random mating within subdivided population.

Table 27. Oocyst rate in the studied households of Kininina hamlet.

Table 28. 2-locus genotypes of oocysts collected from households in Kininina, Tanzania.

Table 29. Comparison of allele frequencies of the MSP-1 gene between Kininina hamlet and Michenga village, and within households of Kininina.

Table 30. Comparison of allele frequencies of MSP-2 within households of Kininina.

Table 31. MSP-1 & MSP-2 of isolates collected on the 21st June 1991, from infected individuals in four households in Kininina, Tanzania.

Table 32 MSP-1 & MSP-2 of parasites extracted from fed mosquitoes collected from one household (8001/1) in Kininina.

List of Figures

Figure 1. Life cycle of malaria parasite

Figure 2. Schematic illustration of the cytogenetics of the life cycle of malaria parasite

Figure 3. Schematic illustration of the sequence structure of the merozoite surface protein-1, MSP-1.

Figure 4. Schematic illustration of the sequence structure of the merozoite surface protein-2, MSP-2.

Figure 5. Map of Sudan

Figure 6. Map of Tanzania

Figure 7. Schematic illustration of the variants of enzymes, GPI, ADA, PEP.

Figure 8. Schematic illustration of variants of the polymorphic proteins detected by 2D-PAGE.

Figure 9. Scheme for identification of allelic serotypes of the MSP-1 antigen using MAbs.

Figure 10. (a) Variants of polymorphic proteins detected by 2D-PAGE of isolate 101/89 mixed with variants of the control clone 3D7. (b) Variants of isolate 111/89 mixed with the variants of the control 3D7 clone.

Figure 11. Serotypes of the MSP-1 within 23 isolates collected from Khartoum in 1988 & 29 isolates from Asar village collected in 1989.

Figure 12. Serotypes of the MSP-2 within 23 isolates collected from Khartoum in 1988 & 29 isolates from Asar village collected in 1989.

Figure 13. Prevalence of variants of the enzymes GPI, ADA & PEP in Asar village during 1989 & 1990.

Figure 14. (a) Serotypes of the MSP-1 within the isolates of Asar during 1989 & 1990. (b) Serotypes of the MSP-2 within the isolates of Asar during 1989 & 1990.

Figure 15. Alleles of MSP-1 of *P.falciparum*, classified by PCR and sequence-specific probes, in blood samples from Asar village.

Figure 16. Alleles of MSP-1 in *P.falciparum* in Asar during 1989 to 1991, classified by size and sequence-specific probes.

Figure 17. Alleles of MSP-2 of *P.falciparum*, classified by PCR and sequence-specific probes, in blood samples from Asar village.

Figure 18. Alleles of MSP-2 of *P.falciparum* in Asar during 1989 to 1991, classified by size (bp) and sequence-specific probe

Figure 19. MSP-2 alleles in primary & recrudescence parasites from patients suspected to have R1 chloroquine resistance.

Figure 20. Mature oocyst on mid-gut surface of a mosquito vector.

Figure 21. Alleles of MSP-2 gene of *P. falciparum*, detected by PCR in blood samples from Michenga village

Figure 22. Alleles of MSP-1, classified by size (bp) and sequence-specific probes, in oocysts from Michenga village.

Figure 23. Alleles of MSP-1 gene in Michenga village, classified by size (bp) and sequence-specific probes. (A) blood forms (B) oocysts.

Figure 24. Alleles of MSP-2 gene, classified by size (bp) and sequence-specific probes, in oocysts from Michenga village.

Figure 25. Alleles of MSP-2 gene of in Michenga village, (bp) and sequence-specific probes. (A) blood forms (B) oocysts.

Figure 26. MSP-2 alleles in *P. falciparum* aquired by fed mosquitoes, identified by PCR and allele-specific probes.

Figure 267 MSP-2 alleles of infected individuals, fed mosquitoes and oocysts in household 8001/1.

Figure 28. MSP-1 alleles in infected individuals in Asar village, Sudan, and Michenga, Tanzania.

Figure 29. MSP-2 alleles in infected individuals in Asar village, Sudan, and Michenga, Tanzania.

Figure 30. Prevalence of sequence variants of the MSP-1 and MSP-2, in Asar, Sudan and Michenga, Tanzania.

List of Abbreviations

ADA	adenine deaminase
bp	Base pairs
Bq	Becquerels
ci	curies
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxyadenosine triphosphate
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
dGTP	deoxyguanosine triphosphate
DAPI	4'-6'-diamino-2-phenylindole
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
ECL	Enhanced Chemiluminescence(3' oligolabelling kit)
EDTA	ethylenediaminetetra-acetic acid
Exp-1	exported protein
EIR	entomological inculation rate
FITC	fluorescein isothiocyanate
GPI	glucose-6-phosphate isomerase
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonicacid)
IFA	immunofluorescence assay
kD	kilodalton
LDH	lactate dehydrogenase
Mabs	monoclonal antibodies
MIC	mininum inhibitory concentration
MSA-1	merozoite surface antigen-1
MSA-2	merozoite surface antigen-2
MSP-1	merozoite surface protein-1
MSP-2	merozoite surface protein-2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue.
NADP	nicotinamide adenine dinucleotide phosphate
PABA	para-amino benzoic acid
PBS	phosphate-buffer-saline
PCR	polymerase chain reaction
PEP	peptidase
PFGE	pulsed field gradient gel electrophoresis
PMMSA	polymorphic major merozoite surface antigen
PMS	phenazine methosulfate
PPO	2,5-diphenyloxazole
RFLP	restriction fragment length polymorphism
RITC	rhodamine isothiocyanate
RPMI	Roswell Memorial Park Institute
SDS	sodium dodecyl sulphate
TCA	tri-chloroacetic acid
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminoethane
UV	Ultra-violet

Chapter 1. Introduction

1.1 Purpose of the study

The objectives of this study have been:-

- (i) To make a detailed genetic characterisation of *Plasmodium falciparum* of Sudan. Prior to 1989, no information on this subject had been available, except for sporadic reports on drug resistance.
- (ii) To determine whether changes in the genetic composition of the parasites in a single community occur over time.
- (iii) To compare the diversity seen in *P. falciparum* in a Sudanese village with that in a Tanzanian village of comparable size but different malaria endemicity.
- (iv) To determine the genetic mechanisms which give rise to the diversity of the parasite types which occur in a small community.

Over the past 15 years, technical developments have made it possible to maintain field isolates of the malaria parasite *P. falciparum* in laboratory culture. This has made it feasible to study polymorphic genetic characteristics of these parasites, such as antigens, enzymes and other proteins (Kemp *et al.* 1990). The molecular basis of many of these characters has been determined (e.g. Tanabe *et al.* 1987; Smythe *et al.* 1990). Recently, development of the polymerase chain reaction (PCR) has made it possible to study variations in certain genes in parasites in fingerprick samples of blood, without the need to culture them (Foley, Ranford-Cartwright & Babiker, 1992).

Natural populations of the parasite have been found to be very diverse, and variant forms of many genes co-exist together in the same population (reviewed by Walliker, 1991). In addition, it has been found that many patients contain mixed infections of more than one genetically distinct *P. falciparum* clone (Thaithong *et al.*, 1984).

Laboratory crossing experiments between unlike clones have shown that mating followed by segregation and recombination of genes into haploid progeny occurs randomly, during the mosquito stage, to generate novel genotypes (Walliker *et al.* 1987; Ranford-Cartwright *et al.* 1993). However, the breeding patterns of natural parasite populations have not been studied systematically.

The high levels of polymorphism existing within natural parasite populations, and the fact that random mating occurs in laboratory crossing experiments, have suggested that panmixia, or random mating, occurs within natural parasite populations (Walliker, 1991). Alternatively it has been suggested that the major reproductive mode is clonal (Tibayrenc *et al.*, 1990). A principal objective of this work has been to study the extent of polymorphism of natural parasite populations, and especially to examine the frequency of cross-mating between genetically different parasites. The study was carried out in two areas of very different transmission intensity (i) the Sudan, where malaria is seasonal and meso-endemic, with transmission confined to a short (10-12 weeks) rainy season, and (ii) Tanzania, where year round transmission occurs, and malaria is classified as holo-endemic.

1.2. Malaria: global view

Malaria is one of the most widespread diseases in the world, being endemic in 102 countries and placing over half of the world's population at risk (World Health Organization, 1980). It represents a major health problem in these countries, affecting 200-300 million people and

accounting for more than one million deaths each year. Africa accounts for half of the cases: at least 300-500 million malaria episodes are treated annually, and between 675,000 and 1 million deaths among children are attributed to malaria (Deming *et al.* 1989; Greenwood, 1990). In many African countries where malaria is endemic it is the leading cause of morbidity, hospitalization and mortality (Kilama, 1993). Children in The Gambia suffer on average one clinical malaria attack per year and about 1600 children die each year from the disease (Greenwood *et al.*, 1987). Malaria represents a risk even in non-endemic countries, through imported cases mainly in returning travellers (Bruce-Chwatt, 1982), or through "airport malaria" from imported infected mosquitoes (Whitfield *et al.*, 1984; Isaacson, 1989).

1.3 The malaria parasites

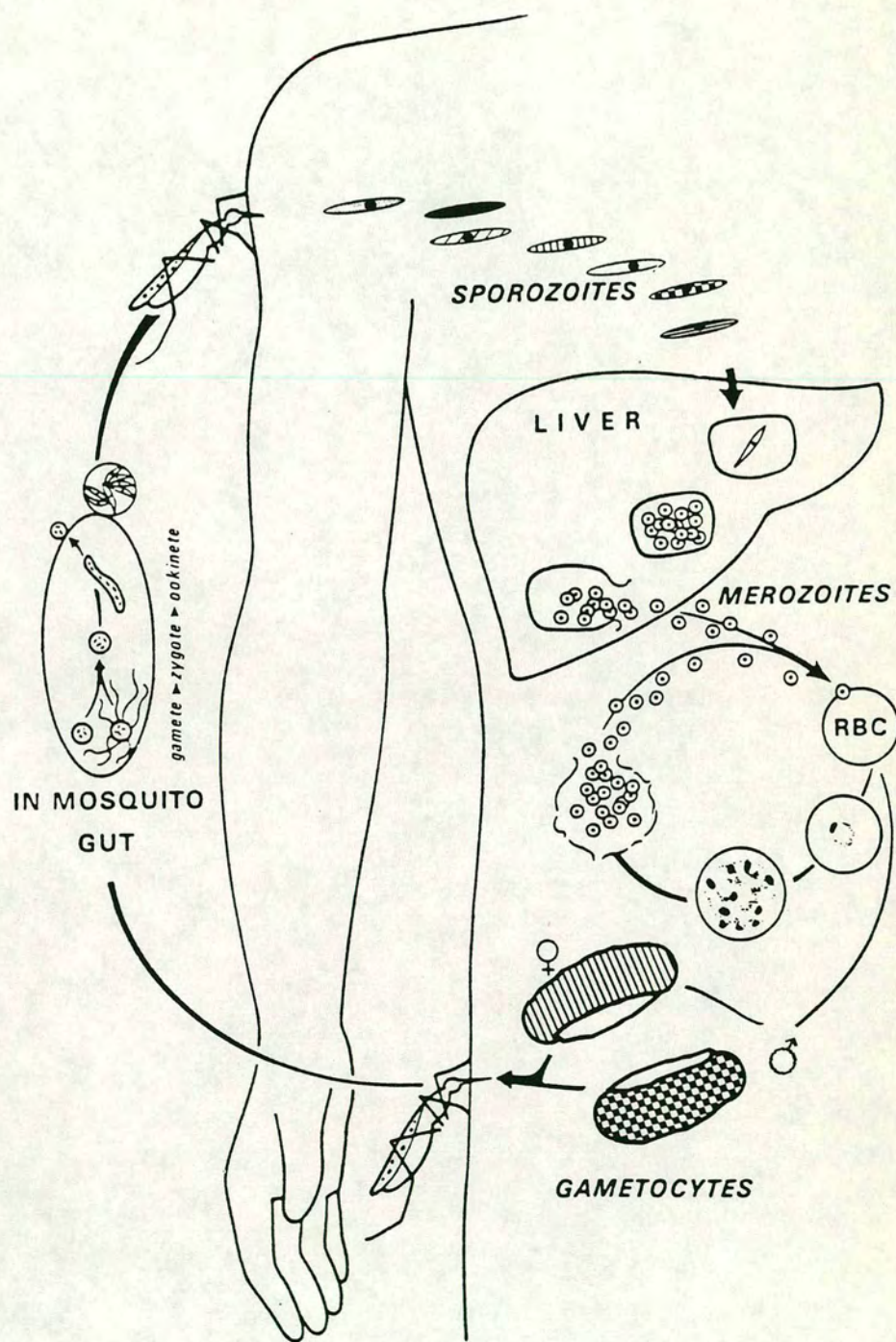
Malaria is caused by protozoan parasites belonging to the genus *Plasmodium*. There are nearly 120 species of *Plasmodium*, four of which infect man: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. They vary in morphology, distribution and pathology. *P. falciparum* is the most common species in tropical and sub-tropical areas.

1.3.1 Life cycle

All four human malaria species share essentially the same general life-cycle (Figure 1). The part of the cycle which occurs in the human host is asexual, although gametocytes are formed in this host. The sexual cycle and sporogony take place in the female *Anopheles* mosquito. Thus gametocytes bridge the two hosts, beginning in the human host and ending in the mosquito midgut.

Figure 1.

Schematic life-cycle of the human malaria parasite *Plasmodium falciparum*. Modified figure reproduced from Hadley, Klotz & Miller (1986). When the mosquito acquires gametocytes of different clones (genotype) represented by different shades in the diagram, two types of zygotes are expected to be produced by selfing of gametes of similar genotypes and one hybrid type by crossing between gametes of the different genotypes. Assuming equal numbers of gametes are produced by each clone and random mating occurs the expected proportions of zygotes will be one of each clone type and two hybrid types (Ranford-Cartwright *et al.*, 1993). Meiosis and recombination produced sporozoites with novel genotypes (Walliker *et al.*, 1987)



Natural infections in man are initiated by the bite of an infected female *Anopheles* mosquito. Sporozoites remain in the circulation for a short period, then within 40 minutes can be detected in hepatic parenchymal cells. Within these cells the parasites divide asexually to form tissue-stage schizonts (exo-erythrocytic schizonts) which ultimately occupy the entire host liver cells. In 7-10 days the infected liver cells rupture and liberate thousands of merozoites which invade red blood cells in the general circulation. Some sporozoites of *P. vivax* and *P. ovale*, but not of *P. falciparum* or *P. malariae*, become dormant in liver cells as hypnozoites which develop into tissue schizonts at a later stage, giving rise to relapses of the infection.

After several generations of erythrocytic schizogony some ring-stage parasites develop into the sexual forms: female macrogametocytes and male microgametocytes. Following ingestion of a bloodmeal by a mosquito they are released from the erythrocytes in the gut and develop into macro and micro-gametes. The process of microgametogenesis takes about 10 minutes, the nucleus dividing into eight portions. Eight flagellated microgametes are formed, each about 20 μm in length and actively motile (Garnham 1988). Fusion of a microgamete with a macrogamete produces a zygote which develops over 24-48 hours into an ookinete, which is motile. This penetrates the midgut wall and rests between the basement membrane of the gut wall and the midgut epithelial cells.

Meiosis occurs within 2 hours of fertilization (Sinden & Hartley, 1985), followed by mitotic divisions (Howells & Davies, 1971). An oocyst develops, where sporogonic mitotic divisions finally result in the production of haploid sporozoites, which become mature between 7 and 15 days from the time of the infective bite. The length of this process depends on the parasite species and the ambient temperature. Mature oocysts measure about 40 to 60 μm . Up to 10,000 sporozoites may be present in a single oocyst (Rosenberg & Rungsiwongse, 1991). The sporozoites are thought to escape through rents in the oocysts and travel in the haemocoelomic fluid to accumulate in the acinal cells of the salivary glands. Following maturation, they are then introduced into a new vertebrate host during a blood feed. The number of sporozoites

which are transmitted per infective bite has been suggested to average only twenty (Ponnudurai *et al.*, 1991) (Figure 1).

1.3.2. Cytogenetic organisation of the *Plasmodium* genome.

Early cytological studies of malaria parasites produced little information about the organisation of the genome and the number of chromosomes at different stages of the life cycle. The principal problem was that the parasite chromosomes do not condense and so can not be visualised by light microscopy (Bahr & Mikel, 1972). However, using electron microscopy, condensed chromatin has been found during the final stages of microgamete formation in the rodent malaria parasite *P. yoelii* (Sinden *et al.*, 1976). During cell division, microtubules with associated kinetochores have been seen. At least ten kinetochores have been counted in individual nuclei of ookinetes. Sinden (1978) estimated that the sexual stages of the parasite had 14 chromosomes. The DNA is thought to be packed into nucleosomes (Wunderlich *et al.*, 1980). Subsequently Prensier and Slomianny (1986) counting the number of kinetochores in the mitotic spindle of young schizonts, confirmed the presence of 14 chromosomes in *P. falciparum*.

The development of pulsed field gradient gel electrophoresis (PFGE) made it possible to separate *P. falciparum* chromosomes on agarose gels (Kemp *et al.*, 1985; Van der Ploeg *et al.*, 1985). This work demonstrated that the genome of *P. falciparum* is organised into 14 chromosomes (Kemp *et al.*, 1987; Wellems *et al.*, 1987), which vary in size from 650 to 340,000 kilobases (kb) (Triglia *et al.*, 1992). Many genes have now been assigned to their chromosomes by blotting and hybridization techniques (Kemp *et al.*, 1990; Walker-Jonah *et al.*, 1992). The sizes of homologous chromosomes differ between different laboratory clones, as well as between freshly collected parasite isolates (Corcoran *et al.*, 1986; Babiker *et al.*, 1991b). This has also been found in other malaria

3.4 mb

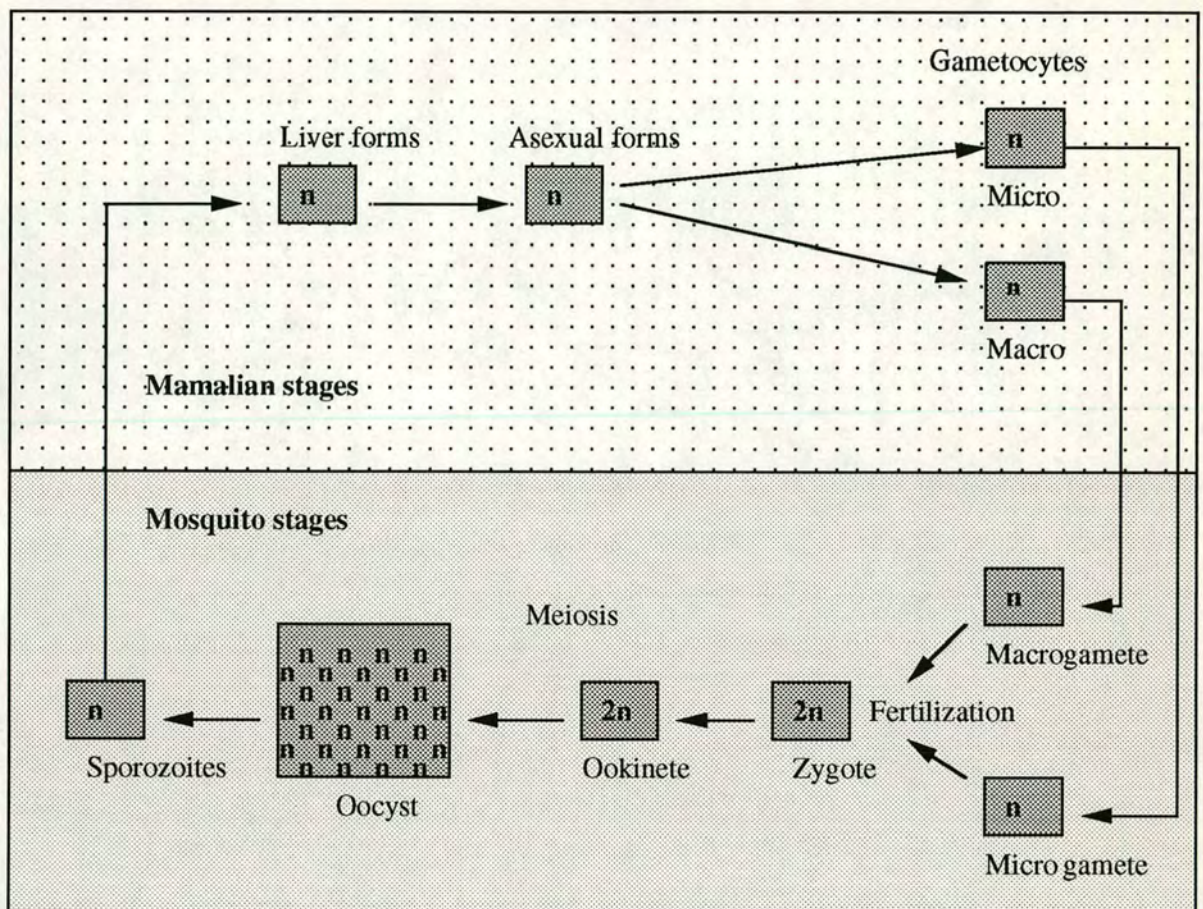


Figure 2.

Schematic illustration of cytogenetic changes in the life cycle of *Plasmodium*. " n " refers to the haploid chromosome complement of the per cell, $2n$ refer to diploid cells. It is assumed that mitotic divisions take place to produce oocysts containing sporozoites.

parasites such as *P. chabaudi*, *P. vinckei* and *P. berghei* (Sharkey *et al.*, 1988; Janse *et al.*, 1986). During mitotic multiplication, DNA rearrangements occur frequently in the subtelomeric regions of the chromosomes (Janse *et al.*, 1993). Size variations in homologous chromosomes of *P. berghei* have been shown to be due to variation in copy number of 2.3 kb repeats, which are generated by either deletions or insertions of this repeat residue (Pace *et al.*, x unib 1990).

Quantitative analysis of the DNA of the blood stages has suggested haploidy of ring-forms, young trophozoites and schizonts (Janse *et al.*, 1986). The only diploid phase in the *Plasmodium* life-cycle is the zygote (ookinete). Meiosis occurs within 2 hours of fertilization (Sinden & Hartley, 1985) resulting in synthesis of approximately 4 times the haploid DNA quantity (Janse *et al.* 1986). These observations are consistent with duplication of the diploid chromosome sets at the first stage of meiosis. Electron microscop. has shown synaptonemal complexes, structures associated with chromosome pairing during the first division of meiosis, in the early ookinete stage (Sinden and Hartley, 1985). The second meiotic division, in which the haploid genome is generated has not been discovered yet. Figure 2 is a schematic illustration of the cytogenetic events during the parasite life-cycle.

1.3.3 Genetics

Genetic studies have provided evidence for the haploidy of the blood forms. This is shown clearly by the detection of only single alleles of genes in cloned parasites. In addition, crossing experiments between parasite clones of the rodent malaria parasite *P. yoelii* and *P. chabaudi* (Walliker *et al.*, 1973; 1975; Rosario *et al.*, 1976) and the human parasite *P. falciparum* (Walliker *et al.*, 1987) have been carried out which have demonstrated that (1) asexual blood forms of the parasites are haploid, (2) alleles of independent genes undergo a Mendelian pattern of inheritance, and (3) recombination between alleles of different genes occurs frequently, due

to re-assortment of genes on different chromosomes (Fenton and Walliker, 1991; Walker-Jonah *et al.*, 1992), and to crossing over between linked genes (Sinnis and Wellems, 1988).

Recently Ranford-Cartwright *et al.*, (1993) have shown that individual oocysts derived from crosses between genetically distinct *P. falciparum* clones include forms containing both parental alleles of two antigen genes, which had derived from cross-fertilization events. The proportions of homozygous and heterozygous forms actually found are in accordance with Hardy-Weinberg expectations of random mating (Ranford-Cartwright *et al.*, 1993). Assuming that equal numbers of gametes are produced by each clone and that random mating occurs, the proportion of zygotes produced are expected to ^{be} one of each parental type, produced by selfing, and two hybrid types, produced by crossing. X 4

1.4. Malaria control strategies.

1.4.1. Malaria control with the vector as target.

In the early 1950s the World Health Organisation (WHO) launched the Global Malaria Eradication Programme, aimed at interrupting the transmission of malaria by spraying homes with insecticide. Elimination of the disease was achieved in some parts of the world where the mosquito populations were relatively small and localised (reviewed by Bruce-Chwatt, 1985). The logistics associated with malaria control were considered beyond the scope of many African countries and large scale eradication was never attempted. However, the excellent results obtained with DDT house-spraying in interrupting malaria transmission encouraged the initiation of more than 20 pilot projects in various African countries. These projects were aimed at complete interruption of transmission as a pre-requisite for malaria eradication. However, within most highly endemic areas the impact was minimal. The Garki project in Nigeria (Molineaux & Gramiccia, 1980) showed that in this area the vectorial capacity was approximately one thousand times the critical value required for maintenance of transmission.

In Garki, the transmission rate was reduced by as much as 90% by the control measures adopted; however, the prevalence of *P. falciparum* decreased by only 25% and returned to its previous level once intensive control had ceased (Molineaux & Gramiccia, 1980).

Recently community-wide use of bed-nets impregnated with insecticide has been adopted as a new vector-targeting control method. The prospects of this approach in Africa are thought to be better than in Central America where multiple insecticide resistance is common and the main mosquito vector *Anopheles albimanus* feeds early in the evening, or in South East Asia where the vectors, *An. dirus* or *An. nuneztovari*, rest out-doors (Curtis, 1993). In contrast, the tropical African vectors, *An. gambiae* and *An. funestus* generally bite late at night and rest indoors to digest their blood meals, so can thus encounter the insecticide. The newly developed synthetic insecticides, pyrethroids, have enhanced the effectiveness of this approach (Curtis, 1993). Data from areas with moderate endemicity have shown significant reductions in cases and/or deaths following the introduction of impregnated bed nets (Alonso *et al.*, 1991). However in holo-endemic areas, it is not clear whether this approach can produce similar results in the absence of regular supplementary drug treatment (Curtis, 1993).

1.4.2. The role of chemotherapy.

The role of chemotherapy in eradication programmes was initially limited to the treatment of individual cases, but as insecticide resistance became widespread, mass drug administration became more commonly used. Following the Alma-Ata conference in 1978, WHO has adopted global primary health care as its strategy, which has prompted the use of presumptive treatment by village health workers (WHO, 1978). It has been agreed that in highly endemic areas, reduction of morbidity and mortality among the two population segments at risk, pregnant women and children up to the age of five years, is a priority for malaria control. The use of combinations of chloroquine and pyrimethamine among children in northern Cameroon reduced parasite prevalence, but after three months it rose again. This was considered to be due to

development of resistance to pyrimethamine (Bruce-Chwatt, 1986). Widespread use of drugs has resulted in increased parasite resistance to most of the drugs in use (Bruce-Chwatt, 1986).

The spread of drug resistant *Plasmodium* has made prophylaxis and treatment increasingly difficult (Bruce-Chwatt, 1986). The earliest report of antimalarial resistance was that of *P. falciparum* to quinine reported from Brazil in 1910 (reviewed by Spencer, 1985) but this was not confirmed by laboratory tests. Apart from Thailand, the clinical response to quinine in other parts of the world remains satisfactory with occasional reports of clinical resistance (Wernsdorfer, 1991). Chloroquine remained the first line antimalarial because of its low cost, efficacy and low toxicity. Unfortunately, in 73 of the 95 countries affected by *P. falciparum* malaria, chloroquine resistance has now been reported (Wernsdorfer, 1991). The frequency and degree of chloroquine resistance is highest in the areas longest affected, and more heterogeneous in areas where it has occurred only recently (Wernsdorfer, 1991). Early reports of resistance to chloroquine came simultaneously from Thailand, and Colombia (reviewed by Peters, 1987). Eventually the two foci geographically expanded and consolidated. In Africa the phenomenon started to develop in the late 1970s (Fogh *et al.*, 1979) and since then it has spread dramatically until, by the late 1980s, it covered most of east, central and west Africa (Payne, 1987). Pyrimethamine/ sulfadoxine (Fansidar) has been used as an alternative drug in areas where chloroquine resistance has been diagnosed. However, resistance to this drug is also well established in many south east Asian countries and in South America. Reports of failure of one or both drugs have now come from many African countries (Bjorkman & Phillips-Howard, 1990; Babiker *et al.*, 1991b).

Much recent research on the chemotherapy of malaria has concentrated on screening natural and synthetic compounds for antimalarial activity. Recently new antimalarials, mefloquine, halofantrine and artemisinin have become available. However, mefloquine resistance has been reported from Thailand and Africa (Bjorkman and Phillips-Howard, 1990). Thus for the

maximum efficacy of chemoprophylaxis as a measure of control, it is essential that there should be an organized effort to monitor the sensitivity of parasites to the drugs in use.

1.4.3. Malaria vaccines

As an alternative approach to control by chemotherapy, a considerable effort has been made in recent years to develop a malaria vaccine. Three sites in the life cycle are proposed for blockage by a vaccine, (a) the sporozoite; if fully effective, an anti-sporozoite vaccine would prevent the parasite developing in the human host and thus could produce sterile immunity, (b) asexual erythrocytic-stages; such a vaccine would be expected to inhibit the replication of asexual blood-stage parasites, and (c) a transmission-blocking vaccine, which would operate by inducing immune responses in the human host that block the fertilization of gametes within the mosquito.

It is not the purpose of this thesis to review the numerous antigens, and their genes, proposed as vaccine candidates. However, in considering the likely efficacy of a vaccine, the diversity of such genes which exist in natural populations of *P. falciparum* is obviously a subject of importance. In this work diversity of three antigens has been studied, the merozoite surface proteins (MSP-1) (Holder & Freeman, 1982), MSP-2 (Smythe *et al.*, 1988) and an exported protein (Exp-1) (Simmons *et al.*, 1987). Immunization with purified MSP-1 has induced significant protection in *Aotus lemurinus* monkeys (Siddiqui *et al.*, 1987). The homologue of this gene of the rodent malaria species *P. yoelii* has been shown to elicit some degree of immunity in mice which can partially or completely protect the host against challenge with viable parasites (Freeman & Holder, 1983). Recently a recombinant polypeptide containing a 15 kD fragment of the C terminal of the antigen has been suggested to be the immunogenic part of the protein, (Daly *et al.*, 1993; Ling, Ogun & Holder, 1994). Murine monoclonal antibodies (Mabs) against MSP-2 were found to inhibit *P. falciparum* growth *in vitro*, and this antigen is being considered as potential vaccine candidate (Epping *et al.*, 1988; Saul *et al.*, 1989). A

synthetic vaccine which contains parts of the MSP-1 and other peptides (Patarroyo *et al.*, 1988) is now undergoing a double blind randomised placebo-controlled test, in a holo-endemic area, in a village in Tanzania (Teuscher *et al.*, 1994), near Michenga village where some of the study for this thesis was carried out.

1.5. Genetic diversity of malaria parasites

Most current molecular and genetic studies of protozoan parasites including malaria, are guided by the aim of identifying functional genes, for example, genes controlling responses to drugs and antigens which stimulate immune responses. During the past ten years an enormous amount of information has been obtained in the field of molecular genetics of *P. falciparum* (reviewed by Kemp, Cowman and Walliker, 1990). Many polymorphic loci have been identified and extensive genetic diversity has been shown within natural populations of the parasite (reviewed by Walliker, 1991). Recently the molecular basis of the polymorphism of many antigen genes has been revealed (Tanabe *et al.*, 1987, Smythe *et al.*, 1990). The use of the polymerase chain reaction (PCR) has enabled analysis of these polymorphic genes, especially of genes with variable numbers of tandem repeats (VNTRs) (eg Kimura *et al.*, 1990, Arnot *et al.*, 1993). Moreover, development of methods to isolate parasite DNA from a fingerprick blood sample has made it possible to carry large-scale screening of natural parasite populations using this method (Foley, Ranford-Cartwright & Babiker, 1992). Alleles of genes on different chromosomes have been found to exhibit a Mendelian pattern of inheritance (Walliker *et al.*, 1987). Thus identification of alleles of such unlinked genes has provided a very useful tool for surveys of natural populations of the parasites, and for studying recombination in these populations.

1.6. Polymorphic genetic characters used in this study

Early work on rodent malaria parasites laid the ground work for genetic characterisation of *P. falciparum*. Electrophoretic forms of enzymes and responses to antimalarials were used extensively to study variations between the rodent malaria species, *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* (reviewed by Beale, Carter and Walliker, 1978). These and other biochemical and molecular characters have also been used to study polymorphisms in *P. falciparum* clones and isolates (reviewed by Walliker, 1985). These other characters include antigens detected by monoclonal antibodies (MAbs), proteins detected by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), restriction fragment length polymorphisms (RFLPs) of genes (eg. Walker-Jonah *et al.*, 1992), sequence-specific DNA probes (Peterson *et al.*, 1988b) and differences in sizes of homologous chromosomes (reviewed by Kemp *et al.*, 1990). More recently analysis of differences in the sequences and sizes of genes with VNTR regions have provided a powerful technique to identify allelic polymorphism in such genes in *P. falciparum* (Kimura *et al.*, 1990; Marshall *et al.*, 1994).

In this study the characters studied were enzymes and other proteins, antigens and drug responses. In addition, alleles of the genes determining two polymorphic merozoite surface proteins denoted MSP-1 and MSP-2 have been characterised using the polymerase chain reaction (PCR) and allele specific oligonucleotide probes.

1.6.1. Enzymes

The early work on rodent malaria parasites showed that electrophoretic variants of enzymes are stable genetic markers which can be used to distinguish species and subspecies of these organisms (Carter, 1978). Some enzymes were found to be highly polymorphic within the studied populations: among 23 isolates of *P. chabaudi* 6 forms of the enzyme lactate dehydrogenase (LDH) were identified (Beale, Carter and Walliker, 1978). In *P. falciparum* the

enzymes glucose phosphate isomerase (GPI), lactate dehydrogenase (LDH), adenine deaminase (ADA) and peptidase (PEP) are particularly useful markers (Walliker, 1985). The first *P. falciparum* populations to be studied enzymatically were in The Gambia (Carter and McGregor, 1973) and Tanzania (Carter and Voller, 1975); subsequently variations in the frequencies of alleles of these enzymes have been observed in different countries (Sanderson *et al.*, 1981; Thaithong *et al.*, 1981; Creasey *et al.*, 1990).

1.6.2 Proteins detected by 2D-PAGE

In this technique radio-labelled parasite proteins are separated according to their iso-electric point and molecular weight on two dimensional polyacrylamide gels (Tait, 1981; Fenton *et al.*, 1985). Not less than 14 proteins can be identified which are polymorphic between different *P. falciparum* clones. The functions of most of the proteins remain to be established.

1.6.3. Antigens

1.6.3.a Merozoite surface protein number 1, MSP-1.

Many *P. falciparum* antigens have now been studied by gene cloning and sequencing. Of particular importance is a family of proteins located on the surface of the merozoites, known as merozoite surface proteins (MSP). The first molecule of this family to be characterised was in the rodent malaria species *P. yoelii*. The antigen was shown to possess some protective properties when used to immunise mice (Holder and Freeman, 1981). A homologous protein has been found in *P. falciparum* (Holder *et al.*, 1985) *P. vivax* (Gibson *et al.*, 1992) and the rodent malaria species *P. chabaudi* (Deleersnijder *et al.*, 1990).

In *P. falciparum* the protein is denoted MSP-1, and is encoded by a single gene located on chromosome 9 (Kemp *et al.*, 1987). MSP-1 has been given various names by different

authors., e.g precursor to the major merozoite surface antigen, PMMSA (Holder *et al.*, 1985; Peterson *et al.*, 1988b) p190 (Tanabe *et al.*, 1987), MSA-1 (Snewin *et al.*, 1991) and gp195, 195 kD glycoprotein (Weber *et al.*, 1986).

MSP-1 is synthesized in the late stages of asexual maturation and becomes a significant surface protein of merozoites. It is thought to be attached to the outer face of the parasite membrane by a glycosyl phosphatidyl inositol anchor (Holder *et al.*, 1985). The protein ranges in size from 185 to 205 kD (McBride, Newbold and Anand, 1985). Before release of merozoites from schizonts, the protein is cleaved into a series of smaller fragments. These fragments form a complex located on the surface of free merozoites (McBride & Heidrich, 1987; Holder *et al.*, 1987). The N terminal fragment is shed from the merozoite surface around the time of invasion (Holder, 1988). A smaller fragment at the carboxy-terminal of 42 kD is further cleaved into two fragments of approximately 34 kD & 19 kD, on the surface of the merozoite (Holder *et al.*, 1987; McBride and Heidrich, 1987; Blackman *et al.*, 1991). The smaller fragment (16 to 19 kD) is retained by the merozoite after invasion of a new host cell (Blackman *et al.*, 1990). The other fragment (33 kDa) is shed (Blackman *et al.*, 1991). The 19 kDa, carboxy-terminal fragment contains a series of cysteine residues which are highly conserved (Daly *et al.*, 1992). A recombinant polypeptide containing the smaller carboxy terminal fragment (15kD) was found to produce complete or partial protection of mice against challenge with *P.yoelii* (Daly & Long, 1993). Thus, this fragment is considered to be a potential vaccine candidate.

The polymorphic nature of MSP-1 among different *P. falciparum* isolates has been demonstrated using a panel of monoclonal antibodies (Mabs) (McBride *et al.*, 1985; Conway & McBride, 1991). Some Mabs recognise epitopes on naturally processed fragments of the molecule, and thus the epitopes can be approximately localised on the molecule (Holder, 1988; McBride and Heidrich, 1987, Lyon *et al.*, 1987). An alternative approach to identify the location of the epitopes of the MABs involved the production of recombinant proteins

representing a known sequence of the protein (Blackman *et al.*, 1993). The epitopes recognised by these Mabs have also been determined by correlation of serological and sequence polymorphism (Conway and McBride, 1991).

Polymorphism in MSP-1 has also been analysed by comparison of DNA sequences of the gene from different parasite isolates and clones (reviewed by Miller *et al.*, 1993). This has revealed that certain regions of the gene are conserved between all alleles analysed so far, while other regions are semi-conserved, and others are highly variable. The molecule has been divided into 17 different blocks based on variations in the degree of polymorphism of different domains (Tanabe *et al.*, 1987)(Figure 3). Each MSP-1 allele so far examined has essentially only one of two alternative combinations of blocks 6-17 (Tanabe *et al.*, 1987).

A polymorphic domain known as block 2 near the N terminus of MSP-1 includes variable numbers of tandem repeats encoding repeating tripeptides, which may vary with regard to both number and sequence in different alleles (Tanabe *et al.*, 1987; Peterson *et al.*, 1988b; Jongwutiwes, *et al.*, 1992). Sequence variants in block 2 examined so far contain one of three types, denoted K1, MAD20 and RO33 after the isolates from which they were originally obtained (Kimura *et al.*, 1990). Unlike the K1- and the MAD20-type alleles, block 2 of the RO33 lacks tripeptide repeats (Certa *et al.*, 1988). Within the tripeptide repeats of MAD20 and K1 types, some repeating units vary in sequence between alleles of the same type, while others are relatively conserved, especially at the 5' and 3' ends of the repeat regions (Jongwutiwes, *et al.*, 1992).

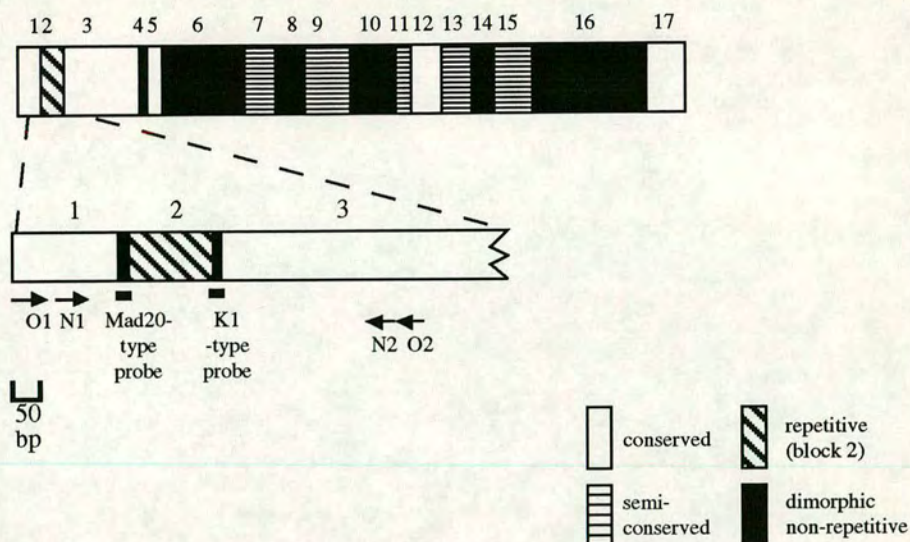
originally
wellcome

Figure 3.

Diagrammatic structure of the merozoite surface protein, MSP-1. The polymorphism in the molecule is shown schematically by dividing the molecule into 17 blocks each of which is conserved, semi-conserved or polymorphic, according to Tanabe *et al.* (1987)(panel A). Block 2 contains polymorphic tripeptide repeats. The number and sequence of these repeats varies between alleles. Three type of sequence in this block have been reported, K1-, MAD20- and RO33-type; RO33 lacks a repetitive sequence. Panel B shows six alleles of the K1-type with different number of repeats and variation within these repeats (Jongwutiwes *et al.*, 1992). Panel C shows five alleles belong to the MAD20 type, in which similar size and sequence variations were found (Jongwutiwes *et al.*, 1992).

In the work described in the following chapters, PCR primers around block 2 were used to amplify this region, to increase sensitivity of the PCR, two sets of primers were used, outer (O1 and O2) and internal (nested) primers (N1 and N2)(section 2.6).

A



B

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
CAMP	SAQ	SGT	SGT	SGT	SGT	SGT	SGT	SGT	SAQ	SGT	SGT	SAQ	SGT	SGT	SAQ	SGT	SGT	SGT	SGT
3D7	SAQ	SGA	SAQ	SGA	SAQ	SGA	SAQ	SGA	SAQ	SGA	SAT	SAQ	SGP	SGT	SAP	SG	SGT		
B358	SAQ	GS	SGP	SGT	P	SGT	SGP	SGT	SGP	SGT	SGP	SGT							
814A	SAQ	SGT	SGT	SGT	SGT	SGT	SGT	SGP	SGP	SGT									
K1	SGT	SGT	SGT	SGT	SGP	SGP	SGT												
B608	SGT	SGT	SGT	SGP	SGT														

C

	1	2	3	4	5	6	7	8	9	10	11	12	13
BND1A	SKG	SVA	SGG	SGG	SVA	SGG	SGG	SVA	SGG	SVA	SGG	SVA	SGG
Wellcome	SKG	SVA	SGG	SGG	SVA	SGG	SVA	SGG	SVA	SGG	SVA	SGG	
MAD20/FC27	SAQ	SVT	SGG	SVA	SVA	SVA	SGG	SGG	SVA	SGG			
HB3	SAQ	SVA	SGG	SGG	SVA	SGG	SVA	SGG					
843	SKG	SVA	SSG	SVA	SGG								

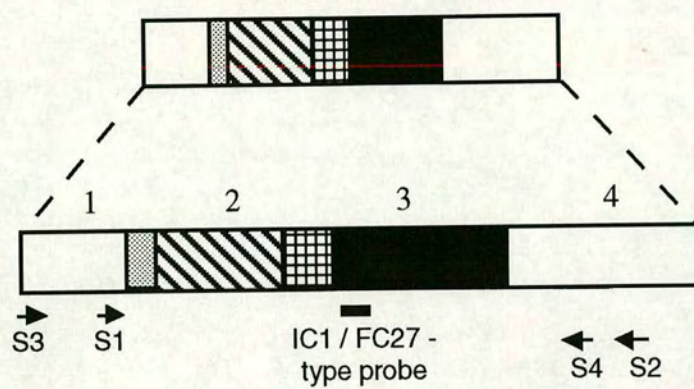
1.6.3.b Merozoite surface protein number 2, MSP-2.

This antigen has been denoted the 35-48 kD antigen (Fenton *et al.*, 1989), or MSA-2 (Smythe *et al.*, 1988). MSP-2 is a distinct membrane protein of approximately 45 kD associated with the merozoite surface. Like MSP-1 it is believed to be anchored to the merozoite membrane by a GPI moiety (Smythe *et al.*, 1988). The gene encoding the protein has been mapped to chromosome number 2 (Kemp *et al.*, 1987). Its size varies between different clones and isolates, between 35-56 kD (Fenton *et al.*, 1989; Clark *et al.*, 1989). Several Mabs have been produced which recognise different regions of the molecule. Some of these Mabs have been found to inhibit parasite growth *in vitro* (Epping *et al.*, 1988). Thus the antigen has been considered as a potential vaccine candidate. Serological studies have shown that there are considerable antigenic differences between the MSP-2 alleles of different isolates (Fenton *et al.*, 1989; Conway *et al.*, 1991).

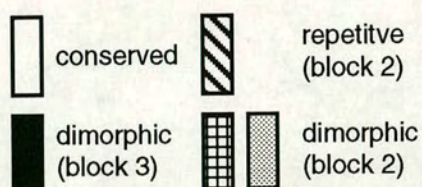
Comparison of sequence data from many isolates and clones has revealed the structural basis of the observed polymorphism (Smythe *et al.*, 1988; 1990; 1991; Fenton *et al.*, 1991, Thomas *et al.*, 1990, Marshall *et al.*, 1994). The gene can be divided into four distinct regions based on differences in the degree of sequence homology between different isolates. The first 43 (block 1) and the last 74 (Block 4) amino acids are common to all clones and isolates studied so far with the exception of one point mutation. However, there is extensive sequence diversity within the central region of the gene (Figure 4). Block 2 contains variable numbers of tandemly repeated sequences. Block 2 can be classified into two groups on the basis of sequence. The IC1 family group is characterised by a short (glycine-serine-alanine rich) sequence repeat (4-8 residues). The corresponding area of FC27 family is not glycine-serine-alanine rich, but contains one or more repeated copies of a 32 amino acid sequence. Following this repeat region, there is an area of a second repeat region of 12 amino acids within the block of FC27-type alleles. Block 3 is characterised by non-repetitive variable sequence that

Figure 4.

Diagrammatic representation of allelic polymorphism of the merozoite surface protein-2 (MSP-2) (Thomas *et al.*, 1990). According to sequence homology of the alleles studied so far, the gene can be divided into 4 blocks. Blocks 1 and 4 are highly conserved. and Block 2 consists of polymorphic repeat sequence, block 3 contains variable regions the sequence of which can be divided into two distinct families, FC27 and IC1 (Smythe *et al.*, 1991). In the work described in other chapters, PCR primers around blocks 2 & 3 were used to amplify these regions. To increase the sensitivity of the PCR, two sets of primers were used, outer (S2 and S3) and nested primers (S1 & S4)(section 2.6).



50
bp



can be classified into either of two sequence types, denoted as IC1 and FC27 (Thomas *et al.*, 1990). These two families correspond to serogroups A and B of Fenton *et al.* (1989). The non-repetitive variable sequence of block 3 flanked the repetitive region. Intragenic recombination in block 2 has been suggested to generate 'hybrid' alleles (Marshall *et al.*, 1991).

1.6.3.c Other polymorphic antigens

Several other antigens of *P. falciparum* have been shown to be polymorphic. Three of these, an exported protein (Exp-1), the circumsporozoite protein (CSP) and the glutamate rich protein (GLURP), will be mentioned further in this thesis and are described briefly here.

Exp-1 is an exported protein localised at the parasite surface of the ring form, the parasitophorous vacuole membrane and the membranes of the vesicles within the erythrocyte cytoplasm in trophozoites (Simmons *et al.*, 1987). Sequence comparison of alleles of several isolates has shown that the gene sequence is very conserved. Only two sites of amino acid substitution have been found, one of which is associated with reactivity to Mab 5.1 (Simmons *et al.*, 1987). Parasites with aspartic acid at position 136 are recognised by the Mab 5.1, and substitution of aspartic acid with glycine abolishes this reactivity (Simmons *et al.*, 1987). Natural isolates have been found to vary considerably with regard to this mutation (Babiker *et al.*, 1991a).

The circumsporozoite protein (CSP) is a major malaria sporozoite surface antigen, and an important candidate for a malaria vaccine (Lockyer *et al.*, 1989). T-cell epitopes of the CS protein have shown a high degree of polymorphism due to non-synonymous mutations in these regions of the molecule (Lockyer & Schwartz, 1987). Non-synonymous mutations in non-repetitive regions of the CS protein gene and other malaria surface antigen genes have been suggested to be a result of immune selection creating diversity in T- and B-cell epitopes within natural *P. falciparum* populations (Lockyer *et al.*, 1989; Arnot, 1989).

GLURP is a glutamate rich protein (Borre *et al.*, 1991) of approximate size 220 kD. The protein is expressed in the parasitophorous vacuole of the liver schizont, the erythrocytic schizont and the surface of released merozoites. Sequence data of some isolates has indicated a high degree of overall homology between alleles of different isolates. However, the gene includes two regions of repeats, R1 and R2. The first (R1) consists of 6 repeats, each containing 15 or 16 amino acids, and two repeat units of 50 amino acids. The second (R2) consists of 14 repeat units of 19 or 20 amino acids residues. PCR analysis of the second region has shown considerable size polymorphism of different alleles in different isolates (Borre *et al.*, 1991).

1.6.4. Variation in drug susceptibility

Resistance to the antimalarial drugs, chloroquine and pyrimethamine, is due to stable gene mutations (Padua, 1981; Rosario, 1981; Peterson *et al.*, 1988a). Pyrimethamine is an antimalarial which acts selectively by inhibiting the enzyme dihydrofolate reductase (DHFR). Sequence data from pyrimethamine resistant and sensitive clones have indicated a role for amino acid 108 in the mechanism of resistance (Peterson *et al.*, 1988a). They have found that in the resistant parasite asparagine is present at this position, while in sensitive parasites either serine or threonine is present. These polymorphisms can be identified using the PCR technique (Zolg *et al.*, 1990). However, genes controlling responses to chloroquine have not been identified. Parasites resistant to chloroquine expel the drug rapidly and reduce the accumulation of the drug in the acid vesicles (Krogstad *et al.*, 1987). The discovery that verapamil reverses this phenomenon *in vitro* led to a proposal that efflux may involve an ATP-driven P-glycoprotein pump similar to that in mammalian multidrug-resistant (mdr) tumour cell lines. *P. falciparum* has been found to have *mdr* gene homologues, (Foote *et al.*, 1989; Wilson *et al.*, 1989), polymorphisms in one of which (*pfmdr1*) have been suggested to confer chloroquine resistance (Foote *et al.*, 1990). However, screening of field isolates did not support this

hypothesis (Awad-Elkariem *et al.*, 1991). Recently Wellems *et al.* (1991) using a genetic crossing experiment have reported the linkage of chloroquine resistance to a region of chromosome 7. The relevance of this observation to resistance in field isolates has not been confirmed. Phenotypic variants to many antimalarials have been detected between natural *P. falciparum* populations (reviewed by Payne, 1987; Thaithong *et al.*, 1983).

1.7. Genetic structure of natural parasite populations.

In studying the population genetics of a parasite such as *P. falciparum*, it is important to consider how diverse alleles of genes are generated and spread among the parasite populations. All genetic polymorphism originates in mutations of individual genes; such novel alleles are then maintained in the population by appropriate selective forces. New alleles may spread into different geographical areas by gene flow among the parasites, or by population movement of their host(s); in *P. falciparum* an example of such gene flow is that of mutant forms of genes conferring resistance to drugs into populations of drug sensitive parasites (Payne 1987). Finally recombination between genes at different loci in the genome occurs during sexual reproduction in the mosquito (Walliker *et al.*, 1987). This gives rise to novel parasite genotypes containing new combinations of genes, which themselves may survive or be lost by natural selection.

A great deal of variation has been found in natural parasite populations of malaria parasites, which takes the form of a continuous gradation of genotypes. The existence of such diversities of genotypes can be attributed to genetic recombination and segregation of genes during sexual reproduction. Clear evidence of genetic diversity has been shown within natural populations of the parasite, by the variant forms of many biochemical markers, enzymes, proteins detected by 2D-PAGE electrophoresis and antigens as discussed in section 1.6. As the major objective behind many of the molecular genetic programmes has been the eventual development of a protective vaccine, the molecular basis of polymorphism of many antigen genes has obvious

importance. Surveys of many parasite populations have now established the fact that considerable heterogeneity occurs within local parasites.

Studies on allelic frequencies of certain *P. falciparum* genes have revealed geographical differences between parasite populations (Creasey *et al.*, 1990). The frequencies of alleles of certain genes have been found to be stable characteristics of a given parasite population from one year to another (Conway *et al.*, 1992). The dynamics and genetic structure of natural parasite populations can thus be studied using such polymorphic genes. The local interbreeding unit (the fundamental unit of the parasite population within which changes in allele frequencies occur) is expected to be small where populations have a clonal structure. Alternatively in populations undergoing random mating it could be limited by factors such as the mosquito flight range and feeding habits as well as other selective factors. Knowledge of the genetic structure of natural parasite populations can provide estimates of ecological parameters which might influence strategies for treatment and control.

In *P. falciparum*, the prolonged use of certain antimalarials has resulted in widespread development of drug resistant parasites. Possible candidate genes responsible for chloroquine resistance have been proposed (Foote *et al.*, 1990; Wellems *et al.*, 1991), although a clear association between these genes and phenotypic resistance has not been shown. Nevertheless, such an approach could lead to identification of characteristic polymorphisms linked to gene or genes controlling responses to antimalarials. *In vitro* responses to pyrimethamine have been demonstrated to be genetically determined (Walliker *et al.*, 1987), and phenotypic resistance has been suggested to result from a single amino acid change in the dihydrofolate reductase-thymidylate synthase gene (Peterson *et al.*, 1988a; Zolg *et al.*, 1990). The maintenance and spread of such polymorphisms among natural populations remains to be studied. This could provide important information about the rational use of this drug for control and treatment of malaria in a particular region.

Early work on enzyme diversity in *P. falciparum* parasites in hyperendemic regions of tropical Africa, The Gambia and Tanzania, showed the presence of more than one variant of the enzymes, GPI, PGD and LDH. These variants coexisted in each region, and different combinations of variants of each enzyme were identified among the isolates examined (Carter and MacGregor, 1973; Carter and Voller, 1975; Sanderson *et al.*, 1981). Similar conclusions were drawn from studies of populations of the parasite in Thailand (Thaithong *et al.*, 1981). Later surveys of parasite populations from different geographic areas revealed that extensive genotypic diversity existed in each region, and variations in allele frequencies of the characters occurs between them (Creasey *et al.*, 1990). Most recently, Conway and McBride (1991) surveyed parasites from The Gambia for variants of three antigen genes and found a very high degree of allelic polymorphism. About 35 different alleles of MSP-1, 8 of MSP-2 and 2 of Exp-1 were detected within a single region. Genotype frequencies were in accordance with those expected if random mating was occurring among the parasites.

PGD not defined

Among other malaria species, *P. vivax* populations in India have been found to be diverse, and variant forms of polymorphic enzymes were distributed randomly within different isolates (Joshi *et al.*, 1989). Natural populations of the rodent malaria species *P. chabaudi* in the Central African Republic have been studied for variation in electrophoretic forms of polymorphic enzymes. Different variants of each of the studied enzymes were found. Out of 12 possible combinations (genotypes) of the studied loci, 9 different genotypes were found. The 3 combinations not found all involved a rare form of one enzyme (Beale *et al.*, 1978).

An important finding of all studies on natural malaria infections has been that multiclonal infections with more than one genetically distinct parasite clone are common (Thaithong *et al.*, 1984; Carter & McGregor, 1973; Creasey *et al.*, 1990; Conway *et al.*, 1991; Babiker *et al.*, 1991a).

1.7.1 Hypothesis of a panmictic structure of *P. falciparum*

In nature *P. falciparum* populations appear highly heterogeneous (section 1.7). The human blood forms are haploid and multiply asexually. Sexual forms develop later from the asexual forms, infect the mosquito vector. An experimental cross between genetically different clones has shown that genes undergo recombination following zygote formation and meiosis in the mosquito (Sinden & Hartley, 1985; Walliker *et al.*, 1987). Malaria parasites undergo an obligatory sexual reproduction phase during the life cycle. Laboratory experiments involving crosses between two genetically different clones of *P. falciparum* have shown that both selfing and crossing between gametes occurs. The genotype frequencies of oocysts obtained in such crosses are in accordance with Hardy-Weinberg expectations for random mating between gametes (Ranford-Cartwright *et al.*, 1993). Similarly Rosenberg *et al.* (1992) have produced evidence that mating between *P. vivax* parasites is also random.

These laboratory findings provide explanations for the enormous diversity of genotypes seen in natural malaria parasite populations (section 1.7). Surveys of such populations have suggested that unlinked genes of the parasite are randomly assorted, suggesting that the parasites are in state of random mating (Carter and McGregor, 1973; Conway and McBride, 1991).

Walliker (1985; 1989; 1991) has emphasised that wild populations of malaria parasites are potentially panmictic, and during the natural course of transmission recombination generates new genotypes frequently. The finding that similar variants of characters occur in different endemic areas globally (Creasey *et al.*, 1990), suggests that *P. falciparum* can be considered as essentially a single interbreeding population. However, local interbreeding units which are geographically isolated exist, within which changes in allele frequencies could occur. This could ultimately lead to the evolution of adaptive characters, and eventually to subspeciation.

1.7.2. Hypothesis of clonal structure

Tibayrenc and colleagues (1990) have suggested that the breeding structure of protozoan parasites, including malaria parasites, is clonal (Tibayrenc *et al.*, 1990; 1991). According to this hypothesis sexual reproduction (segregation and recombination) is rare within natural populations of these parasites. The hypothesis is based on their claim that only a limited number of genotypes occur within a natural parasite population. While there is good evidence that parasites such as *Trypanosoma cruzi* and *Leishmania* species have such a clonal breeding structure, the idea that *P. falciparum* was also likely to be in this category was a surprising one, especially because sexual reproduction is an essential part of the life cycle of malaria parasite.

Other support for the clonality hypothesis comes from the work of Read *et al.* (1992), who claim that the occurrence of female-biased gametocyte sex ratios in natural *Plasmodium* populations is an indication of frequent self-mating. According to the hypothesis of these workers, genes encoding female-biased sex ratios will be favoured by natural selection. Thus, they suggest that there is a dominant genotype in the gametocyte population and that a considerable frequency of selfing is occurring in natural populations. Day *et al.* (1992) have also proposed that in nature single genotype infections occur at a higher frequency than would have been expected in panmictic populations. Both authors have emphasised that the frequency of cross-mating required to generate departure from panmictic populations remain to be addressed. Recently Maynard Smith *et al.* (1993) described an intermediate type of population genetic structure, called the epidemic, in which population reproduce sexually but appear superficially clonal due to ecological isolation. Thus, the authors suggested that the evidence shown by Day *et al.* (1992) as an indication for clonal propagation in *P. falciparum* could be explained by such an epidemic pattern of reproduction.

1.7.3. Implication of population genetic structure on the epidemiology and control of malaria.

Organisms that undergo genetic exchange and recombination are capable of producing far greater numbers of new genotypes than clonally reproducing organisms. This has significant implications for efforts to control malaria. For example, if a malarial vaccine is to achieve a maximum protective covering, the allelic diversity of the candidate antigen genes will need to be taken into account. If a vaccine is based on a cocktail of antigens whose genes are unlinked, reassortment of alleles of these genes during meiosis will produce numerous new gene combinations. A further source of variation is likely to be intragenic recombination, giving rise to new alleles of antigen genes (Tanabe *et al.*, 1987; Marshall *et al.*, 1991, Conway *et al.*, 1991; Kerr *et al.* [submitted for publication]). This would occur at meiosis following cross-fertilization of gametes containing unlike alleles of the gene concerned.

With regard to control efforts based on anti-malarials, genetic studies have shown that responses to some anti-malarials are clearly genetically determined in laboratory crosses (Walliker *et al.*, 1987). As discussed above (section 1.6.4.) polymorphisms that are associated with the responses to some anti-malarial drugs have been determined. The maintenance and spread of such polymorphisms among natural populations depends on the rate of genetic exchange within these populations. In a panmictic population, gene flow occurs readily unless geographic and ecological obstacles exist. On the other hand, the clonal model (Tibayrenc *et al.*, 1990) implies that treatment and diagnosis will depend on the genetic characteristics of these discrete genetic units, or "clonets" (Tibayrenc *et al.*, 1991). In the panmictic model such discrete units do not exist and many numbers of different genotypes could be expected to occur.

1.8. Aim of the thesis

The main aim of this thesis is to investigate in detail allelic polymorphisms and genetic structure of some natural *P. falciparum* populations. In Chapter 3, the genetic diversity of *P. falciparum* populations in Sudan is studied. The parasite population of a village (Asar) is studied in detail. Chapter 4 describes a longitudinal survey (three years) to assess the impact of the seasonal pattern of transmission on parasite population. In Chapter 5, the heterogeneity of the parasite population's response to antimalarials e.g. Chloroquine, pyrimethamine and mefloquine is examined. In addition, the prevalence of resistance to chloroquine and pyrimethamine is monitored over two transmission seasons. In Chapter 6, the population genetic structure of *P. falciparum* in Michenga village, Tanzania is discussed. Chapter 7 describes the proposed spatial structure of parasites in households of farms in a hamlet near Michenga village, Tanzania. Finally, chapter 8, the diversity seen within *P. falciparum* in Asar is compared to that in Michenga village, Tanzania, which is similar in size but with a markedly different level of malaria endemicity.

L.c.

Cays

Chapter 2. Material and Methods

2.1. Study areas

2.1.1. Asar village, eastern Sudan

Asar village is in the eastern region of Sudan (Figure 5), approximately 18 km south-east of Gedaref, the principal town of the area which lies between the Atbara river in the North, the Rahad river in the south and the Ethiopian border in the East. Approximately 4000 inhabitants live in the village. The villagers practice traditional subsistence agriculture centred around sorghum (Dura) cultivation, which is dependent upon seasonal rainfall (July-October). The annual rainfall in the area ranges between 300 to 700mm.

Malaria transmission is seasonal, peaking during and after the rainy season which is from September to November. *A. arabiensis* is the main mosquito vector and *P. falciparum* is the predominant malaria species (Haridi, 1972; El-Gadal, 1986).

2.1.2. Khartoum, central Sudan.

The capital, approximately 5 to 7 million inhabitants, is in an area where malaria transmission is seasonal following the rainy season, August to November. However, irrigated farms around the Blue and the White Nile keep a small focus of perennial transmission in areas near these farms, e.g. Elgeref West. The main mosquito vector is *A. arabiensis* and *P. falciparum* is the major malaria species (Wernsdorfer and Wernsdorfer, 1967; El-Gadal, 1986).

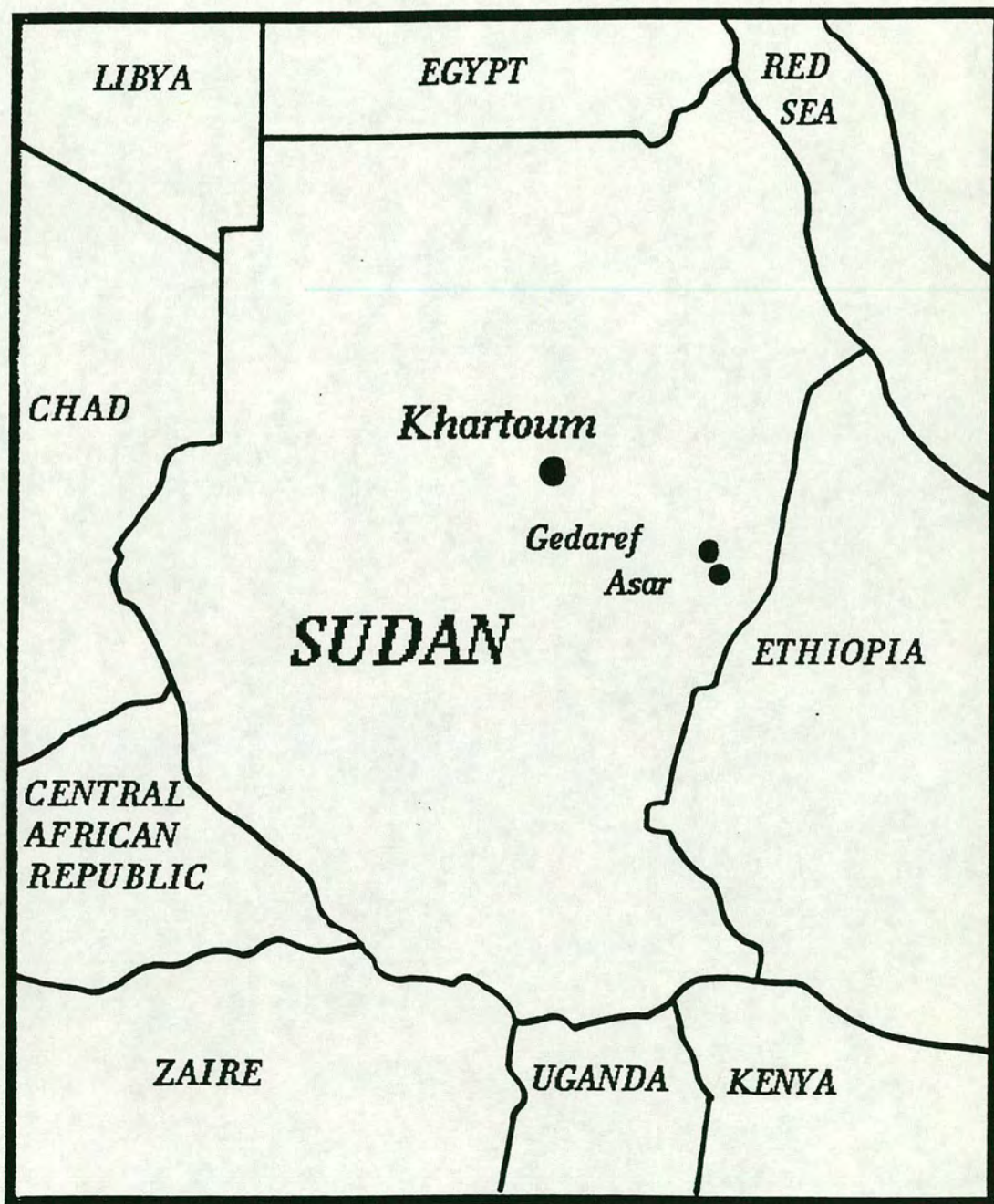


Figure 5.

Map of Sudan showing Asar village and the study area in the eastern region.

2.1.3. Michenga village, south east Tanzania

Michenga village is located in Kilombero District, within the Morogoro Region of southeast Tanzania (Figure 6). Topographically, the district extends along the Usagara and Uzungwa mountains in the northwest and the Kilombero River in the south. The annual rainfall ranges between 1,200mm to 1,800mm. The population of the village is approximately 3000 inhabitants. The area is holo- to hyper-endemic for malaria, with peak transmission in June and July. The main malaria species is *P. falciparum*, which accounts for 95% of all malaria infections, and the main mosquito vectors are *A. funestus* and *A. gambiae* (Tanner *et al.*, 1991; Smith *et al.*, 1993).

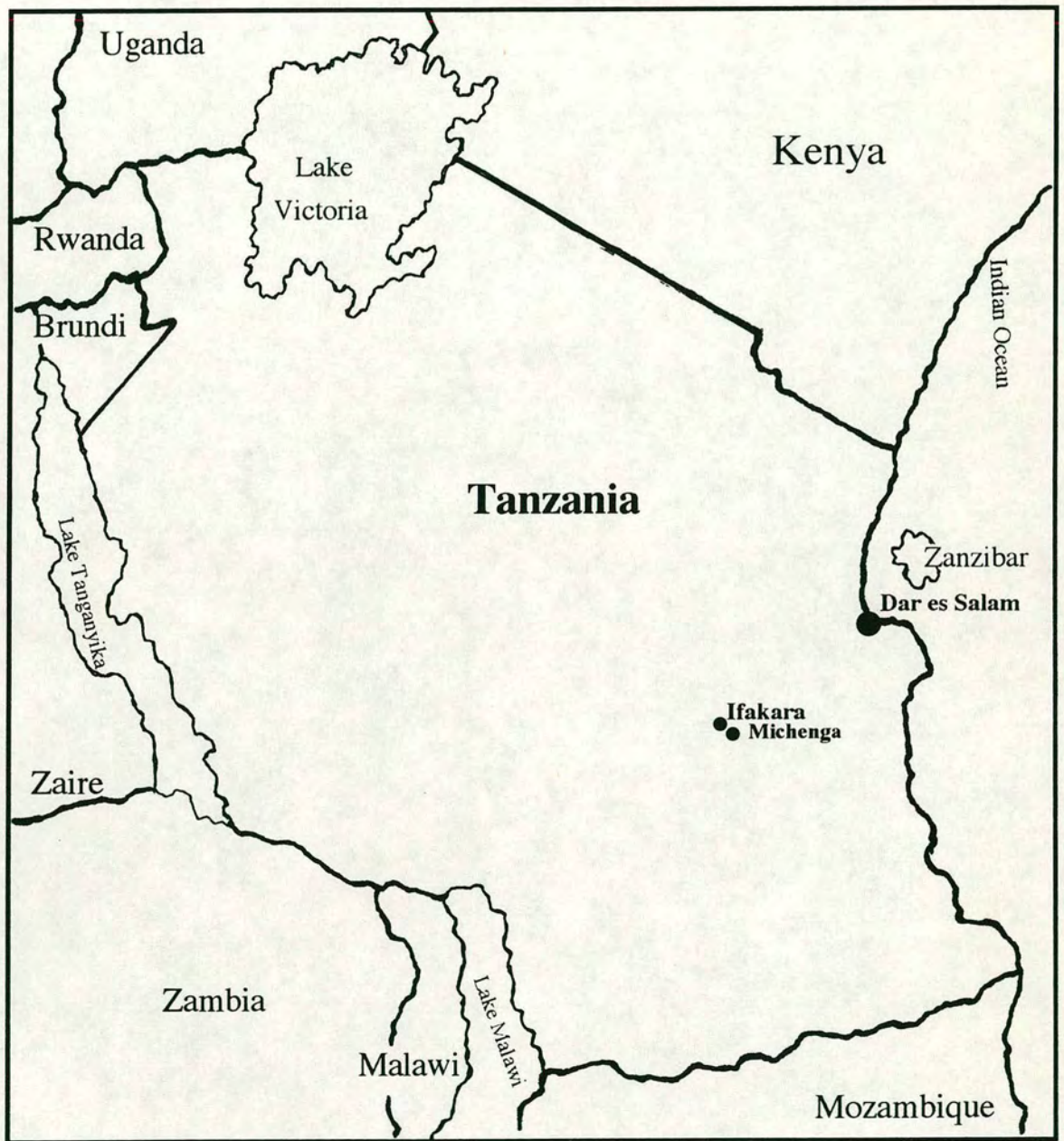


Figure 6.

Map of Tanzania showing Michenga village and the study area in south east Tanzania.

2.2. Collection of samples of *Plasmodium falciparum*.

2.2.1. Diagnosis of *Plasmodium falciparum*

Thick and thin blood smears were stained with Giemsa stain according to the WHO specifications (1983). For staining, the stock Giemsa solution (BDH) was diluted 1:9 with phosphate buffer pH 7.2 (0.2% Na₂HPO₄, 0.14% KH₂PO₄). Dried thin films were fixed by methanol for a few seconds. Thick films were not fixed. Slides were then allowed to stain for 30 minutes. *P. falciparum* was morphologically distinguished from other *Plasmodium* species, using the WHO Bench Aids for the Diagnosis of Malaria (WHO, 1985).

2.2.2. Collection of blood samples

Blood samples were collected from patients who had a pure *P. falciparum* infection. In Michenga village the following method was used. The middle finger was cleaned with 70% ethanol, and wiped with dry cotton wool. A sterile lancet was used to puncture the skin, and the first drop of blood was removed with dry cotton wool. 500 to 700 µl of blood was then collected in a capillary blood tube containing lithium heparin (Microvette, Sarstedt).

In Asar village and Khartoum blood samples were collected from patients attending clinics. 5 ml of venous blood were collected from each patient using vacutainer tubes (Becton Dickinson Vacutainer System) containing lithium heparin. Samples were kept at room temperature until transferred to a small laboratory in Gedaref for further processing.

2.2.3. Collection of mosquitoes

An. gambiae and *An. funestus* were collected in Michenga village using the indoor resting method (Molineaux *et al.*, 1988). Fed mosquitoes were collected from inside houses in the early

morning with the help of aspirators, and kept in an insectary in small cages for 5-7 days to allow oocysts to develop. During this period the mosquitoes were fed with a 5% glucose solution. Each mosquito was then dissected and the midgut examined microscopically for the presence of oocysts. Midguts containing only single oocysts were collected for this study.

2.3. Preservation and *in vitro* cultivation of *P. falciparum*

The blood samples collected were first preserved by deep-freezing them in liquid nitrogen. Subsequently, following transport to Edinburgh, they were established in *in vitro* culture.

2.3.1. Freezing and storage of *P. falciparum* isolates.

Parasites collected from both villages were cryopreserved shortly after the blood was collected using a modification of the method of Aley *et al.* (1984). Parasites from Asar village were chosen for cryopreservation on the basis of high parasitaemias, (approximately 3-5%) and healthy morphology. The blood samples in the vacutainers were first transferred to 15 ml polystyrene centrifuge tubes (Falcon), and centrifuged at 2000g for 5 minutes. Plasma and buffy coat were removed, and the cells washed three times in at least 10x the volume of the packed cells in sterile incomplete RPMI medium (section 2.3.3). The final supernatant was discarded, and the volume of the packed cells was measured. An equal volume of cryopreservative solution (28% glycerol, 3% sorbitol, 0.65% NaCl) was mixed with the cells by adding the solution dropwise and gently mixing the cells by Pasteur pipette. This mixture was left for 5-10 minutes, and then 0.3-0.5 ml aliquots were transferred to ampoules (Nunc), which were placed directly in liquid nitrogen. Samples were then transported in liquid nitrogen containers to Edinburgh.

2.3.2. Thawing of deep-frozen *P. falciparum* isolates.

The method was essentially as described by Aley *et al.* (1984). Each isolate was established in culture by removing the required ampoule from liquid N₂, and thawing in a 37°C water-bath. The cryopreserved mixture was transferred to a 15ml sterile polystyrene tube. The volume of the mixture was measured and 0.2 ml of 12% NaCl solution was added for each 1 ml of the red blood cells cryopreservative/mixture. The solution was added dropwise while mixing the blood cells with a Pasteur pipette. This new mixture was allowed to stand for 3 minutes, then 1.6% NaCl was added, 10 ml for each original 1 ml of cryo-mixed blood, dropwise with constant mixing. The mixture was spun at 2000g for 5 minutes, the supernatant was removed, 0.9% NaCl, 0.2% dextrose solution was added in a similar way, 10ml being used for each original 1 ml of blood. The mixture was spun at 2000g for 5 minutes, the supernatant was removed, the cells resuspended in complete RPMI medium at 5% haematocrit and placed in an incubator at 37°C.

2.3.3. RPMI 1640 culture medium

RPMI 1640 (Gibco) is the medium of choice for *P. falciparum* culture. 10.4g of powdered medium, is added to 900 ml. of distilled water with 5.4g HEPES (Sigma) buffer and the volume is adjusted to 960 ml. The medium is then sterilized by filtration through a 0.22 mm pore membrane, dispensed into 100 ml aliquots and stored at 4°C until used. Two types of media were used

- (a) Complete medium, in which the RPMI 1640/HEPES solution is supplemented with 4% v/v of 5% sterile sodium bicarbonate, human serum 5-10 ml/100 ml and 50mg/ml gentamicin sulphate (Sigma).
- (b) Incomplete medium, was made to the same formula without the serum.

2.3.4. *In-vitro* culture of *P. falciparum* isolates.

Following removal from the deep-freeze, isolates were cultured in flasks, using a modification of the methods of Trager and Jensen (1976) and Haynes *et al.* (1976). To culture thawed isolates, cultures were established in flasks in a 5 ml culture volume at 5% haematocrit, and gassed with a mixture of 1% oxygen, 3% carbon dioxide and 96% nitrogen. Approximately 2×10^6 cells/ml of complete medium of mouse peritoneal wash cells were added to enhance parasite growth (Trenholme and Philips, 1989). Fresh red blood cells were added every three days, and the complete medium in each flask changed every day. The parasites were grown for several asexual cycles (approximately 1-2 weeks), until the parasites were adapted to the *in-vitro* conditions and parasitaemia was increasing exponentially. A thin film was made every day and the level of parasitaemia determined by counting the number of viable parasites/10000 erythrocytes. An appropriate dilution with freshly washed erythrocytes was made for each culture when it had reached a parasitaemia of 4-5%. Cultures were used for different experiments at different parasitaemias and stages of growth.

2.4. Cloning of *P. falciparum* isolates

Cloning was performed using the limited dilution method described by Rosario (1981) which was briefly as follows. Since the cloning method depends on the isolation of single red cells infected with single parasites, cultures were shaken for 48 hours prior to cloning to reduce the proportion of cells infected with multiple parasites. The cloning procedure was started when not more than 10% of the red cells in a culture had multiple parasite infections. The parasitaemia was precisely determined, and the number of erythrocytes per ml was counted in a cell chamber. Finally, cultures were diluted to give 0.5 parasite per 100 μ l using 5% haematocrit fresh red blood cells in complete RPMI. Diluted cultures were then pipetted into 96-well microtest plates, 100 μ l in each well. The plate was placed in a gassed flow chamber and incubated at 37°C. Medium was changed every 48 hours. On days 6 and 12, 100 μ l of red

cell at 5% hematocrit were added to each well and the contents of each well were then split into two wells. Smears of cultures were taken on day 14 and 21. On detection of growing parasites, the contents of the well were transferred to a flask and established in culture as described above.

2.5. Characterization of *P. falciparum* isolates for enzymes, proteins, drug response and antigens.

2.5.1. Enzyme electrophoresis

Cultured isolates were examined for the activity of the enzymes glucose phosphate isomerase (GPI), adenine deaminase (ADA) and peptidase (PEP). The method was basically as described by Carter and McGregor (1973) and Sanderson *et al.* (1981)

2.5.1.1. Preparation of parasite material.

Parasites were prepared by concentration of parasites freed from their host cells by lysis with saponin. Cultures with a high parasitaemia containing trophozoites and schizonts were first transferred to centrifuge tubes and spun at 2000g for 5 minutes. The supernatant was removed, and the pellet volume was determined. 0.15% saponin in incomplete RPMI medium was added, at a volume three times that of the pellet, and mixed thoroughly. Lysis of the red cells occurred almost immediately. To avoid lysis of parasites, the culture/saponin mixture was diluted immediately with incomplete RPMI medium at three times the volume of the mixture. The preparation was spun at 4000 rpm for 10 minutes, and the supernatant and red cell ghosts were pipetted off. The freed parasite material was then washed with incomplete RPMI medium and pelleted by centrifugation at 4000 rpm for 10 minutes. Following two further washes, parasite material was either examined for enzyme activity immediately or stored at -20°C until required

2.5.1.2. Cellulose acetate electrophoresis (CAE)

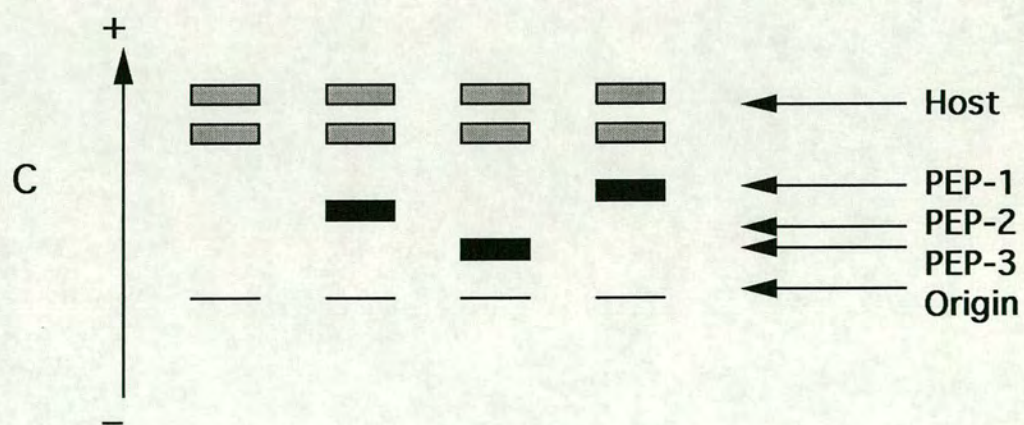
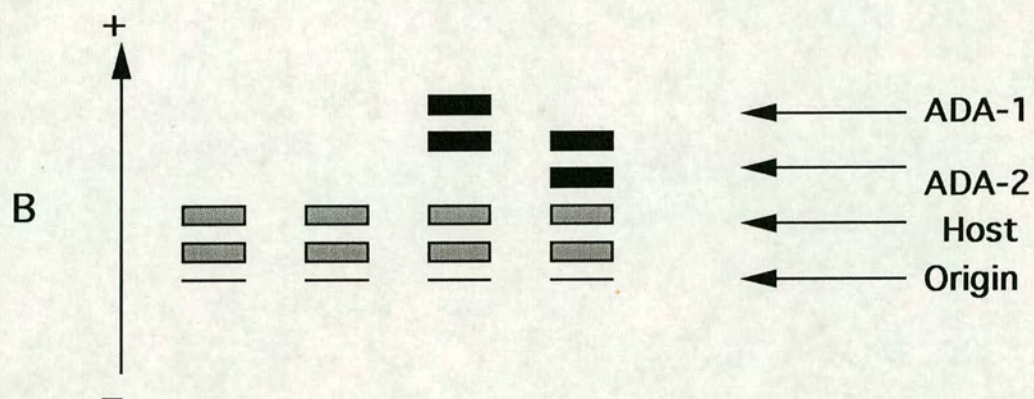
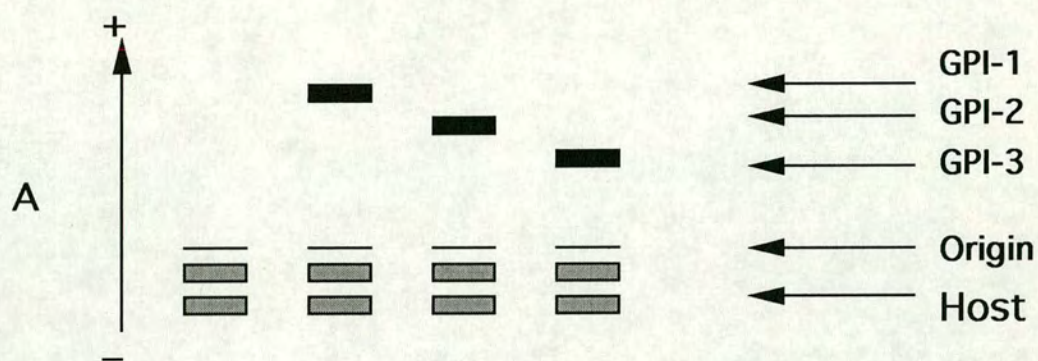
Parasite material was lysed with a small quantity of distilled water. Electrophoresis was then carried out on cellulose acetate electrophoresis plates (Titan ISO-VIS), soaked in Supra-heme buffer (Tris-EDTA-Boric Acid pH 8.2-8.6) according to the manufacturer's instructions (Helena Laboratories). Following electrophoresis, plates were incubated with the appropriate enzyme staining solution (Table 1). The variants of different enzymes were determined according to their electrophoretic mobility in reference to known samples (Figure 7). The human host itself usually possesses equivalent enzymes to the parasite, which may affect the interpretation of the results. Thus, an uninfected blood sample is run parallel to parasite samples.

Table 1. Electrophoretic conditions and staining solutions for the enzymes, GPI, PEP and ADA.

Enzyme	Running condition		Assay solution	
	voltage	time		
GPI	300v	15 min	Buffer (0.5M Tris--HCl pH 8.0)	2ml
			Fructose-6-phosphate	25g
			NADP	5mg
			MTT	5mg
			Glucose-6-phosphate dehydrogenase	0.01ml
			PMS.	2mg
ADA	300v	7 min	Buffer (0.1M phosphate pH 7.5)	2ml
			Adenosine	10mg
			MTT	5mg
			Xanthine oxidase	0.01ml
			Nucleoside phosphorylase	0.01ml
			PMS	2mg
PEP	300v	15 min	Buffer (0.02M citrate- -phosphate pH 5.5)	2ml
			leucyle-leucine acetate	10mg
			snake venom	3mg
			peroxidase	3mg
			MnCl ₂	10mg
			O-dianisidine	2mg

Figure 7.

Schematic illustration of variants of the enzymes, GPI, ADA and PEP of *P.falciparum* and human host, following electrophoresis in cellulose acetate gels (Sanderson *et al.*, 1981).



2.5.2. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE).

The method used was essentially that of Fenton (1985). Radio-labelled parasite proteins were first separated in a tube gel according to their charge. They were then separated by their mobility through a polyacrylamide gel with SDS to enable each protein to be separated according to its particular molecular weight.

2.5.2.1. Incorporation of radio-labelled methionine into parasites.

P. falciparum isolates were first established in culture. When parasitaemia had reached 4-5%, and with a majority of ring-stage forms, the culture medium was replaced with methionine-free EMEM medium (Eagle's minimal medium modified with Earle's salt) (Gibco) which lacked methionine, para-amino-benzoic acid (PABA) and glutamine, but supplemented with HEPES (5.94 g l⁻¹), PABA (1 mg l⁻¹), hypoxanthine (50 mg l⁻¹), reduced glutathione (0.6 g l⁻¹) and glucose (2g l⁻¹). 50 µCi (1.85mBq) of [S³⁵] methionine (Amersham) was added to each 5ml of parasite culture. The cultures were then incubated overnight at 37°C.

2.5.2.2. Preparation of parasites for electrophoresis

After approximately 18 hours incubation, red cell free parasite material was prepared by saponin lysis. This was achieved by subjecting the parasitised cells to treatment with saponin at low concentration (section 2.4.1.2). The resulting parasite pellet was digested using lysis buffer (2%NP40, 9.5M urea, 2%ampholine, 5%mercaptoethanol). Incorporation of methionine into protein was monitored by liquid scintillation counting. 2x10⁵cpm were applied to each gel.

2.5.2.3. 2D gel electrophoresis.

(a) First dimension

The first dimension was carried out in an iso-electric focussing tube gel. 9 M urea was mixed with ampholines, pH 5-7 and pH 3.5-10 in a ratio of 4:1, 4% w/v acrylamide and polymerising reagents (0.01% w/v ammonium persulfate and 0.007% v/v TEMED). The gel was poured to a height of 11 cm in the 13 cm length X 1.5mm diameter tubes, which were then sealed with parafilm. The gel solution was then covered with overlay buffer (8M urea) and allowed to set for 1 hour. The overlay buffer was then removed and replaced with 20 µl lysis buffer overlaid with distilled water. Gels were left again for one hour.

At this stage the gels were ready for prefocussing. The water and lysis buffer were removed and replaced with fresh lysis buffer. Parafilm was removed from the bottoms of the tubes and anodal buffer (0.01M H_3PO_4) was added to cover them. Then the cathodal buffer (0.02M NaOH) was overlayed on the lysis buffer. Prefocussing was carried out at 200V for 15 minutes, 300V for 30 minutes and 400V for 30 minutes respectively. Following this, the overlaid lysis buffer and the cathodal buffer were removed from the top of the tubes, the parasite samples were added, overlaid with sample overlay buffer (2% v/v ampholines and 4M urea) and left to focus for 14-16 hours at 400V. Gels were removed using water pressure through a syringe. They were then incubated for 1 hour on a shaker in equilibration buffer (10% glycerol v/v, 5% mercaptoethanol (v/v), 2.3% SDS (w/v), 0.125M Tris pH 6.8), to remove ampholines and urea and to allow SDS to bind to proteins.

(b) Second dimension gel

The resolving gels were 10% polyacrylamide (0.375 Tris, pH8, 10% acrylamide w/v, 0.1% SDS w/v, 0.032% ammonium persulfate w/v, 0.0002% TEMED v/v.). The solution was

mixed and poured between two glass plates to a height of 12cm. The gels were overlaid with 0.1%SDS and left to polymerise. An upper stacking gel(0.11M Tris, pH 6.8, 4.3% acrylamide w/v, 0.1%SDS w/v, 0.036% ammonium persulphate w/v, 0.0005% TEMED v/v) was poured on top of the resolving gel to concentrate proteins into a tight band. This was overlaid with 0.1%SDS and allowed to polymerise. The apparatus was set and electrode buffer (25mM Tris, 0.191 M glycine and 0.1%SDS) was added. The first dimension tube gels were then attached to the top of the resolving gels using 1% agarose in equilibration buffer. Electrophoresis was carried out overnight at 6 V/ gel. Tracking dye (0.1% w/v bromophenol blue in equilibration buffer) was used to check the progress of the electrophoresis.

2.5.2.4. Fluorography

For fluorography the method used was essentially as described by Laskey and Mills (1975). Following SDS-PAGE proteins were fixed in 50% TCA. The gels were rinsed for 30 minutes with constant agitation in dimethyl sulfoxide (DMSO, Sigma) to dehydrate them. This step was repeated three times, followed by three hours of impregnation with PPO (2,5 diphenyloxazole,) using 22.2% (w/v) solution in DMSO. Then gels were washed thoroughly in distilled water for one hour. They were then dried under vacuum on an LKB gel drier, exposed to Amersham MP films in cassettes and incubated at -70°C for 7-14 days.

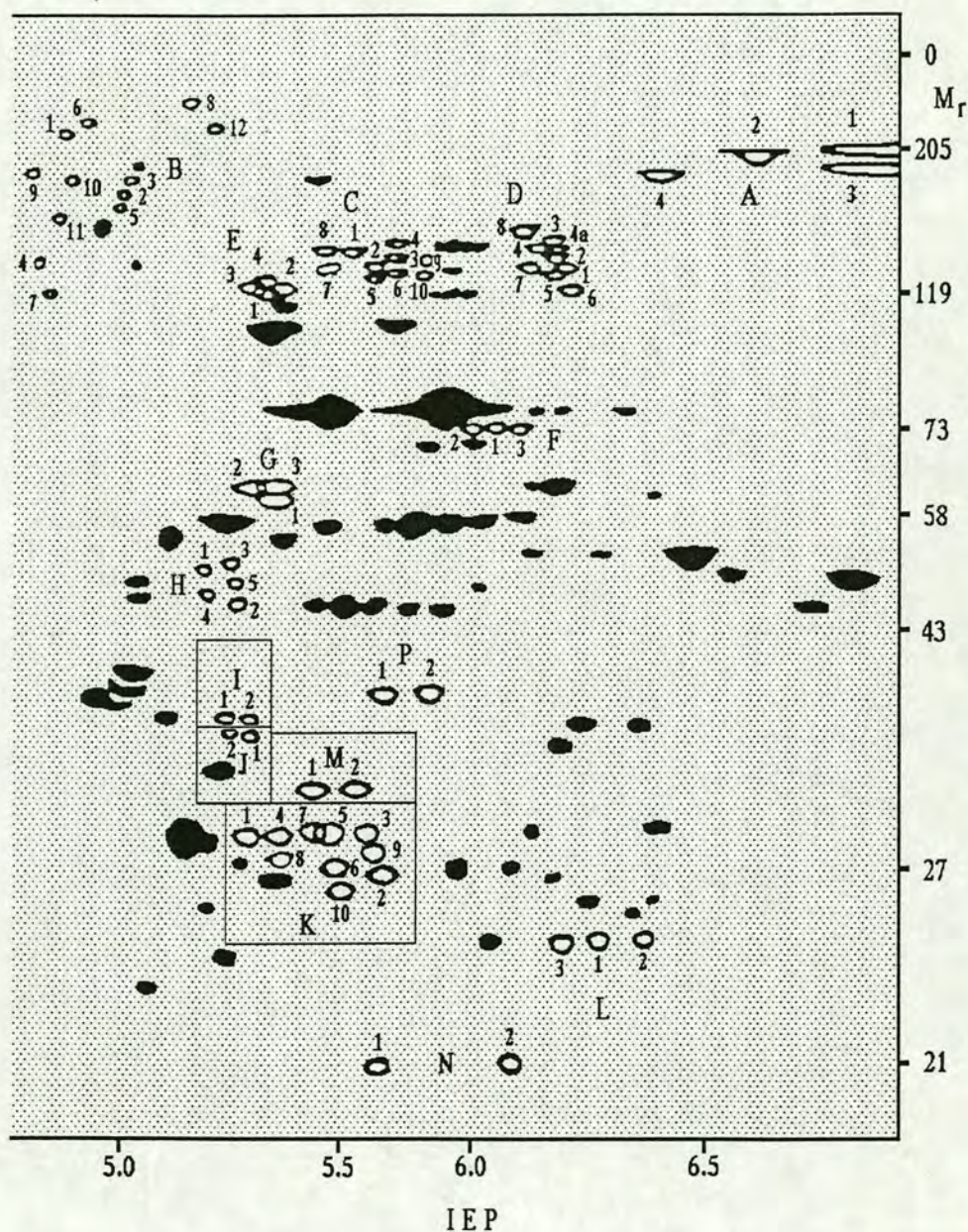
2.5.2.5. Identification of polymorphic proteins.

Following autoradiography different proteins were identified according to their locations on the 2D gel, as described by Fenton *et al.* (1985). Each polymorphic protein was designated by a different letter and different alleles of each protein were denoted by different numbers (Figure8). Standard *P. falciparum* clones of known protein types are mixed with the isolates to help identifying the unknown proteins (Figure 10).

Figure 8.

Schematic illustration of variants of *P.falciparum* polymorphic proteins detected by 2-DPAGE (reproduced from Babiker *et al.*, 1991).

2D-PAGE PROTEINS OF PLASMODIUM FALCIPARUM



2.5.3. Susceptibility of *P. falciparum* to chloroquine, pyrimethamine and mefloquine.

Isolates were tested for their drug sensitivity using the method of Thaithong *et al.* (1983). The minimum inhibitory concentration (MIC) of chloroquine, pyrimethamine and mefloquine which kills all, or nearly all, the parasites, was determined for each isolate.

2.5.3.1. Set-up of microtiter plates

All isolates were tested for their susceptibility to drugs after growth in culture was considered satisfactory and parasites appeared morphologically normal. The initial parasitaemias of cultures were first determined and then diluted with uninfected blood at 5% haematocrit in complete RPMI medium to a final parasitaemia of 1%. Following dilution, 100 µl of each isolate were dispensed into each well of a row of eight wells of a microtiter plate (Falcon). The plate was then incubated for one hour at 37°C to allow the red cells to settle. Supernatants were then removed and replaced with complete RPMI medium containing a range of drug concentrations. The cultures were incubated for 72 hours during which time the culture medium containing drug was changed every 24 hours. Thin film slides were then prepared from each well, fixed and stained with Giemsa stain and examined microscopically (section 2.2.1.). The presence or absence of parasites was recorded for each drug concentration. In parallel with each set of isolates, standard drug sensitive and resistant control clones, 3D7, HB3 and 7G8, were tested on each plate, to monitor any minor variations in media or drug concentrations.

2.5.3.2. Drug concentrations

At the beginning of each experiment, dilutions of each drug in complete RPMI 1640 medium were prepared and stored at -4°C until required. The following concentrations were used:

(1) Chloroquine sulphate (May and Baker) The following concentrations of chloroquine base were prepared and tested: $0.1 \times 10^{-6} \text{M}$, $0.2 \times 10^{-6} \text{M}$, $0.4 \times 10^{-6} \text{M}$, $0.8 \times 10^{-6} \text{M}$ and $1.6 \times 10^{-6} \text{M}$.

(2) Pyrimethamine base (Wellcome) initially dissolved in dimethyl sulfoxide DMSO (Sigma) The concentrations tested were:- 10^{-8}M , 10^{-7}M , 10^{-6}M , 10^{-5}M and 10^{-4}M

(3) Mefloquine hydrochloride (Sigma) M.W. 412. The following concentrations of mefloquine base were prepared and tested: $0.1 \times 10^{-6} \text{M}$, $0.2 \times 10^{-6} \text{M}$, $0.4 \times 10^{-6} \text{M}$, $0.8 \times 10^{-6} \text{M}$ and $1.6 \times 10^{-6} \text{M}$.

2.5.3.3. Interpretation of the test

The results are expressed in terms of the minimum inhibitory concentration (MIC) of drug, which is the lowest concentration that kills all or nearly all the parasites after exposure to drug for 72 hours. In some cases a small number of parasites appeared to survive a given drug concentration which killed the majority of parasites of a given isolate. In such cases only the lowest concentration in which the majority of the parasites were killed was recorded. The interpretation of this test was considered as arbitrary; however, it proved reproducible for all the isolates and clones tested.

2.5.4. Characterization of three polymorphic antigens using monoclonal antibodies.

2.5.4.1. Preparation of antigen slides

P. falciparum-infected cells in cultures at 4-5% parasitaemia with a high proportion of schizonts were washed three times with incomplete RPMI 1640 medium and resuspended at approximately 10% haematocrit. 25 μl of the suspension was pipetted on to each well of 12-well multispot glass slides (C.A. Hendly Ltd., Essex). The slides were left overnight to dry at room temperature, and stored at -20°C , in sealed polythene bags containing silica gel desiccant (Conway, Greenwood & McBride, 1991).



2.5.1.2. Monoclonal Antibodies (Mabs)

A panel of murine monoclonal antibodies (Mabs) recognising three polymorphic surface antigens of *P. falciparum* was used (Table 2). The antigens were merozoite surface protein-1 (MSP-1) (Holder & Freeman, 1982), merozoite surface protein-2 (MSP-2) (Smythe *et al.*, 1988) and an exported protein, Exp-1 (Simmons *et al.*, 1987) (Table 2.). Mabs to each antigen had been raised in fusions against a number of *P. falciparum* isolates (see references of table 2). Details of the isotype specificity, working dilutions and source of the MAbs are given elsewhere (Conway and McBride, 1991).

2.5.4.3. Indirect immunofluorescence assay (IFA)

The test was performed on the multispot-slide preparations of parasitized blood. Multispot-slides were first removed from the freezer and allowed to warm to room temperature, then immediately fixed in acetone. 30 µl of each MAb was placed on separate wells of the slides and allowed to incubate for 30 minutes. Each Mab was then carefully removed by Pasteur pipette, and the slides washed 3X in phosphate buffer saline (PBS), 10 minutes for each wash. 10 µl of a mixture of 1:50 diluted FITC (fluorescein isothiocyanate)-conjugated rabbit anti-mouse IgG antibody and DAPI (4,6-diamino-2-phenylindole, Sigma Ltd.) diluted 1:1000 was then added to each well, and incubated for 30 minutes. The slides were then washed 3X in PBS (5 minutes each) and a few drops of 9:1 PBS/glycerol mixture added.

A fluorescence microscope was used to visualise parasites, an FITC-fluorescence filter being used to visualise the MABs and a DAPI-fluorescence filter to visualise the parasite DNA. Polymorphic epitopes were identified by the presence or absence of fluorescence of a given MAb.

Some isolates were examined for mixed serotypes using a double labelling technique, in which Mabs of different isotypes were placed together on the same sample and stained with a mixture of isotype-specific FITC-antibody and TRITC (Tetramethylrhodamine isothiocyanate)-antibody (Walliker *et al.*, 1987). The FITC and TRITC were visible under different filters on the microscope.

2.5.4.4. Identification of serotypes at the MSP-1, MSP-2 and Exp-1 antigen.

Serotypes (alleles) of the three antigens were assigned numbers according to their pattern of reactivity with the different MAbs, according to the scheme of Conway & McBride, (1991). 52 possible combinations of the MSP-1 epitopes (Figure 9) and similarly 10 combinations of the MSP-2 epitopes (Conway and McBride, 1991) were generated, although certain have never been found. However, in the Gambia 35 serotypes of MSP-1 and 8 of MSP-2 were observed. Polymorphism in Exp-1 is limited to two serotypes, (5.1+)-type and (5.1-)-type.

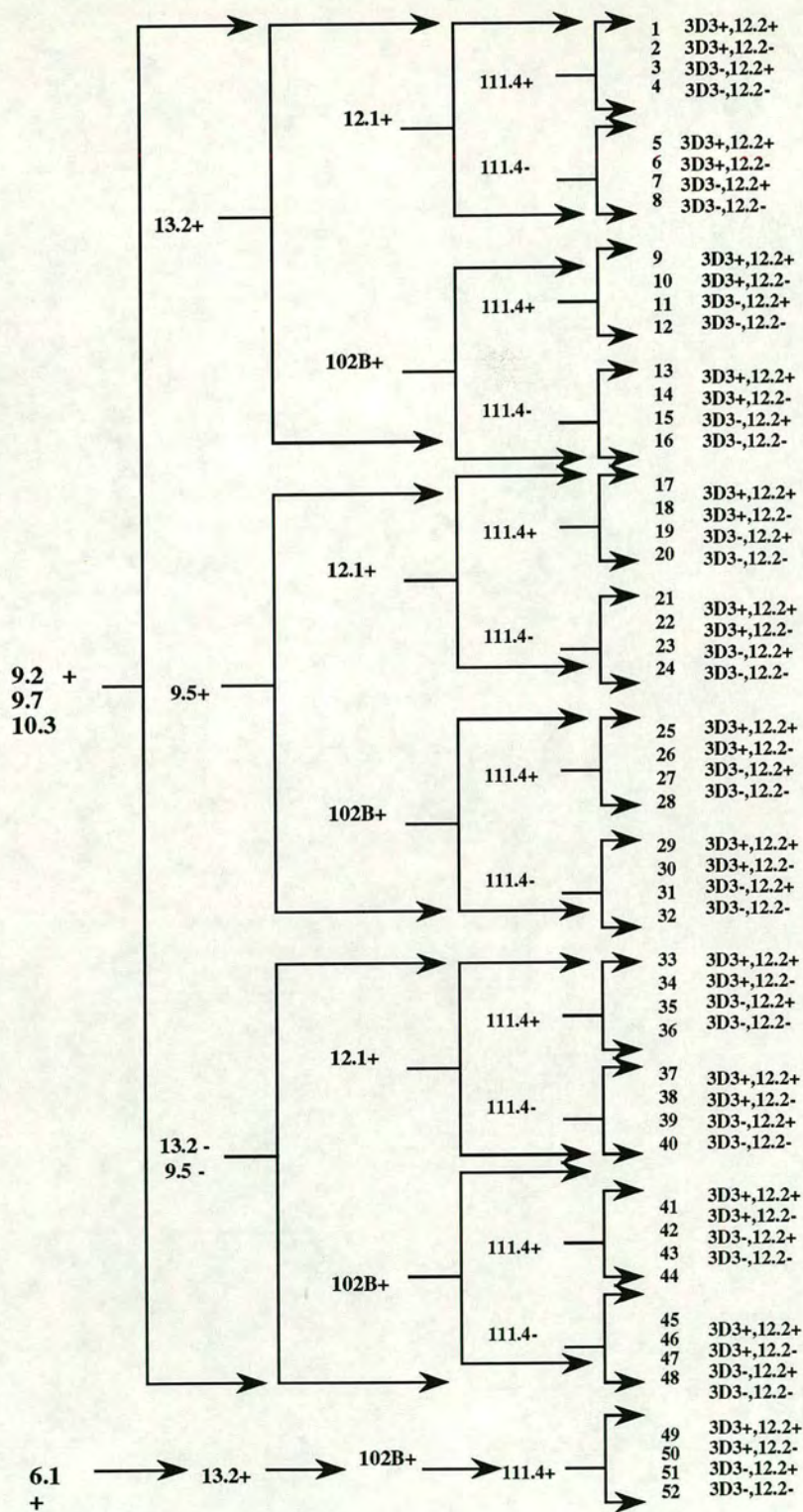
Table 2. MAbs specific for epitopes of MSP-1, MSP-2 and Exp-1 antigens of *P. falciparum*. Isotype specificity and working dilutions are described in Conway and McBride. (1991)

Antibody	location	Reference
MSP-1 MAbs		
9.8-4-4-1	conserved conformation	1
111.4	block 17, 16 kD K1-type	3
6.1-1-3	dimorphic block 16 40kD fragment (K1-type)	1,2
13.1-2	"	1,2
17.1-3	"	1,2
9.2-6-2	dimorphic block, conformational (MAD20-type)	1,2
9.7-1	"	1,2
10.3	"	1,2
7.3	conformational epitope	1,2
35-4	block 13-15, 36 kD fragment	4
1-1C	block, 80K fragment	3
10-2B	block 4, 80K fragment K1-type	5
12.1-5-4	" MAD20-type	5
13.2-3	block 3, 80K fragment K1-type	1,2
9.5-1-5-1	" MAD20-type	1,2
12.3D3.10	block 2, repeats	6
12.2-1-1	"	1,2
MSP-2 MAbs		
13.4-2-1	repeat region, block 2 IC1-type variants	7,8
8-5D	"	7,9
12.3-2-2	dimorphic block 3 IC1-type specific	7,8
12.5-1-2	"	7,8
12.7	"	7,8
8G10/48	repeat region block 2. FC27-type specific	10
8F6/49	FC27 specific	10
Exp-1 Mab		
5.1		11

References : (1) McBride *et al.*, 1985; (2) McBride *et al.*, 1987 (3) Holder *et al.*, 1985 (4) Gentz *et al.*, 1988 (5) Conway *et al.*, 1991 (6) Lyon *et al.*, 1987 (7) McBride personal communication (8) Fenton *et al.*, 1989, (9) Stanley *et al.*, 1985 (10) Saul *et al.*, 1989; (11) Simmons *et al.* (1987). See also diagrammatic representation of polymorphism of MSP-1 & MSP-2 gene, shown in Figs, 3 & 4 respectively.

Figure 9.

Scheme for identification of serotypes of MSP-1, using Mabs (Conway & McBride, 1991). The scheme generates 52 different profiles of reactivity with the Mabs used. Each serotype is defined by a unique reactivity, however, some of these profiles have never been reported. Serotypes which are negative for Mabs 12.2 & 3D3 (serotype, 4, 8, 12 ..etc) can further be divided into two serotypes according to reactivity with other Mabs (31.1, 31.2, & 31.7) (McBride unpublished work, data not shown). Some Mabs (7.3, 13.1, 17.1, 1-1C & 34.5) are not shown in the figure since they share an identical distribution to Mab 6.1.



2.6. Typing of MSP-1 and MSP-2 by Polymerase Chain Reaction (PCR) and hybridization with allele-specific oligonucleotide probes.

2.6.1. Preparation of DNA from parasite cultures

P. falciparum isolates were cultured *in vitro* (section 2.3.4) to a parasitaemia of approximately 4-5%. Free parasites were first prepared from a 5ml culture as described above (2.4.2.1). The parasite pellet was transferred to a 1.5ml microfuge tube, then an equal volume of buffer A (150mM NaCl, 25mM EDTA), was added, mixed gently and centrifuged for 5 minutes. The pellet was resuspended in 0.4ml of buffer A, to which 10 µl of 10% SDS and 50mg of proteinase K were added, then incubated at 55°C for 2 hours. An equal volume of phenol was added, mixed well and centrifuged at 13000 rpm in a microfuge. The supernatant (aqueous layer) was transferred to a fresh tube and an equal volume of a 1:1 mixture of phenol:chloroform was added, mixed, centrifuged at 13000 rpm for 2 minutes and the aqueous layer transferred to a fresh tube. An equal volume of chloroform was added, mixed and spun at high speed for 2 minutes. The aqueous layer was transferred to a fresh tube. 2 times the volume of the aqueous layer of ice-cold absolute ethanol and 120 µl of sodium acetate at pH 5.2 were added. The tube was mixed very gently, and the DNA precipitated by incubating at -20°C for 30 minutes. The mixture was then centrifuged at 13000 rpm for 10 minutes, the supernatant removed and the precipitated DNA washed with 70% ethanol. The ethanol was finally removed, and the pellet freeze-dried in a speedvac (Savant) for 5-10 minutes. Finally the DNA was resuspended in distilled water.

2.6.2. Isolation of DNA from fingerprick blood samples

DNA was prepared from fingerprick blood samples by the method of Foley, Ranford-Cartwright & Babiker (1992). 20-50 µl of packed red cells were added to 500 µl of ice cold

5mM sodium phosphate pH 8.0, vortexed, and centrifuged in a microfuge at 1300 rpm for 10 minutes. The supernatant was discarded and the process repeated three times. A white layer which was mainly red cell ghosts, appeared as a pellet, most of which was removed to improve the quality and quantity of the PCR product. Finally, 50 µl of sterile water was added to the rest of the pellet, vortexed and boiled for 10 minutes. After centrifugation in the microfuge at 1300 rpm for 10 minutes, the supernatant containing the DNA was stored at -20°C or used immediately for PCR.

2.6.3. Extraction of DNA from oocysts and from parasites acquired by fed mosquitoes.

The method used was as described by Ranford-Cartwright *et al.* (1991). Mosquito midguts containing single oocysts were first placed into microfuge tubes containing 50µl of lysis buffer [100mM NaCl, 25mM EDTA (pH8.0), 10mM Tris-HCl (pH8.8), 0.5% sarkosyl] with proteinase K (1mg ml⁻¹). The mixture was incubated at 55°C for 1 hour. 10µl of 10mg/ml salmon sperm DNA and 80 µl of a 1:1 mixture of phenol/chloroform were added to each tube, vortexed briefly, and centrifuged at 1300 rpm for 10 minutes. The aqueous layer was removed to a fresh tube 40 µl of chloroform was added, vortexed briefly and centrifuged at 13000 rpm for 2 minutes. The aqueous layer was transferred into a fresh tube and an equal volume of isopropanol at -20°C was added, vortexed briefly and incubated at -20°C for 20 minutes. The tube was then centrifuged at 13000 rpm for 30 minutes in a cold room. The isopropanol/water mixture was then aspirated off and the pellet washed twice using ice-cold 70% ethanol. The pellet was then dried [speedvac]. Finally 10 µl of sterile distilled water was added to the pellet and boiled for 5 minutes to inactivate nucleases and the proteinase K. The DNA was either used immediately for PCR or stored at -20°C.

2.6.4. Polymerase Chain Reaction (PCR).

All DNA preparations were subjected to PCR amplification (Saiki *et al.*, 1985). using primers flanking polymorphic regions of the merozoite surface antigen genes, MSP-1 and MSP-2 (Table 3, Figures 3 and 4). For MSP-1, the primers recognised conserved sequences of block 1 and 3 flanking block 2 which includes tandemly repeated amino-acids (Figure 3). For MSP-2, primers recognised conserved sequences of blocks 1 and 4 around the central region of the gene, which contains variable sequences and tandemly repeated amino-acids (Figure 4). To increase sensitivity of the PCR, two sets of primers were used, outer and internal (nested) primers (Foley, Ranford-Cartwright & Babiker, 1992; Ranford-Cartwright *et al.*, 1993). Amplified DNA of the first PCR was used as a template for a second PCR amplification using the nested primers.

Table 3. PCR primers used to amplify polymorphic region on MSP-1 & MSP-2. See also figures 3 and 4.

Primer	length(bp)	Sequence 5'to 3'	Position
O1	26	CACATGAAAGTTATCAAGAACTTGTC	5' MSP-1 OUTER
O2	22	GTACGTCTAATTCATTTGCACG	3' MSP-1 OUTER
N1	20	GCAGTATTGACAGGTTATGG	5' MSP-1 NESTED
N2	18	GATTGAAAGGTATTTGAC	3' MSP-2 NESTED
S3	21	GAAGGTAATTAAAACATTGTC	5' MSP-2 OUTER
S2	22	GAGGGATGTTGCTGCTCCACAG	3' MSP-2 OUTER
S1	19	GAGTATAAGGAGAAGTATG	5' MSP-2 NESTED
S4	20	CTAGAACCATGCATATGTCC	3' MSP-2 NESTED

These primers were first described by Foley, Ranford-Cartwright & Babiker (1992) and Ranford-Cartwright *et al.* (1993). See figures, 3 and 4.

2.6.4.1. PCR conditions

DNA was amplified in 20µl of PCR reaction mixes containing PCR buffer (50 mM KCl, 10mM Tris-HCl[pH8.8], 2.5mM MgCl₂, 0.02% gelatin), 100 nM of each primer, 75 µM each of dATP, dTTP, dCTP, dGTP and 0.5 units of AmpliTaq DNA polymerase (Boehringer). Samples were overlaid with mineral oil.

For MSP-1, the DNA was subjected to 30 cycles (94°C/25s; 50°C/35s; 68°C/150s) of amplification performed on a Biometra TRIO thermal block. The conditions and number of cycles were the same for both sets of MSP-1 primers. During the final cycle the extension step at 68°C was continued for 10 min. For MSP-2, DNA was amplified using different conditions for each set of primers. First the outer primers were used and 30 cycles of amplifications at 94°C/25s; 42°C/1min and 65°C/2min were carried out. The second round of amplification using the nested primers was also for 30 cycles, at 95°C/25s; 50°C/1min and 70°C/2min. The extension step, at 65°C or 70°C, was for 10 minutes.

Oocyst DNA was amplified using the same reactions, replacing 10µM of dGTP with the base analogue 7-deaza-2-deoxyguanosine as described by Ranford-Cartwright *et al.* (1991).

2.6.4.2. Agarose electrophoresis.

Following PCR, 10µl of each PCR-amplified product was electrophoretically separated on a 1.6% agarose gel in Tris-Borate-EDTA (0.09M boric acid, 0.09M Tris, 0.002M EDTA) buffer containing ethidium bromide (5µg/ml). DNA molecular weight markers (Boehringer, type VI) were run in parallel tracks. PCR-fragment sizes were then estimated from their distance of migration relative to the size markers.

There are technical difficulties in estimating the sizes of amplified DNA fragments containing variable number of tandemly repeated sequence (VNTR). Measurement errors associated with estimating the length of these fragments are inevitable. As has been shown by workers investigating VNTR loci of other organisms, the estimated fragment length does not specify the number of repeats with certainty (Weir, 1993). To accommodate the continuous nature of fragment lengths of alleles at VNTR loci, methods based on measurement errors and independence of alleles at VNTR loci in human populations have been established (reviewed by Weir 1993). Fragments of closed lengths can be grouped together into discrete "bins" around an estimated average length. Each "bin" may contain several alleles with different numbers of repeat, but for the purpose of population genetic studies can be regarded as a single allele. The alleles based on the amplified region of each of the genes studied in this thesis have been clustered in this way, the "bin" boundaries being defined by the length of the very close fragments. If an estimated length is close to the boundary of a bin, the fragment is assigned to the bin with the highest frequency.

2.6.5. Southern blotting of PCR-amplified fragments.

PCR fragments were transferred from agarose gels to nylon membranes (Genescreen) using the Southern blot technique (Sambrook *et al.* 1989). Briefly, the procedure is as follows. Following electrophoresis, the separated PCR-amplified DNAs were depurinated and denatured. This was accomplished by treating the gel with acid (0.25M HCl) for 15 minutes and alkali (0.2M NaOH, 0.6M NaCl) for 30 minutes. Finally the gel was equilibrated in a neutralising solution (1M Tris, 1.5M NaCl, pH8). Transfer of the DNA fragments to nylon membranes was achieved by capillary diffusion of a high salt solution (20 X SSC [175.32g NaCl, 88g trisodium phosphate]) through the gel and filter into a stack of absorbent towels.

DNA fragments were then covalently bonded to membranes using ultraviolet light. The procedure was essentially as described by Church and Gilbert (1984). After the PCR-fragments

were transferred, the membranes were dipped into 0.4N NaOH for 30-60 seconds to ensure complete denaturation of immobilized DNA, followed by a rinse in neutralising solution (1M Tris, 1.5M NaCl,pH8). The wet membranes were then laid face up on a glass plate, and irradiated for 3-5 minutes with a 254 nm shortwave UV source. The membranes were either used immediately to start pre-hybridization, or dried and stored at room temperature wrapped in filter paper.

2.6.6. Labelling and detection of sequence-specific oligonucleotide probes using the non radio-active, enhanced chemiluminescence (ECL) system.

2.6.6.1. Sequence-specific oligonucleotide probes

The regions of MSP-1 and MSP-2 amplified in this work contain sequences which vary in different alleles. These sequences can be recognised by hybridization of PCR-amplified DNA containing these regions blotted on to membranes with allele-specific oligonucleotide probes. For MSP-1, probes denoted K1, MAD20 and RO33 were used as described by Kimura *et al.* (1990) (Fig.4 & Table 4). Similarly for MSP-2, two new allele-specific probes, IC1 & FC27, were designed for this work based on sequences in the repetitive region of the gene (Smythe *et al.*, 1990)(Fig. 4. & Table 4).

Table 4. Allele-specific oligonucleotide probes for the MSP-1 and the MSP-2 gene.

Name	Gene	length	Mr	sequence
K1	MSP-1	27	8928	GCATCAGCTGGAGGGCTTGCACCAGAT
MAD20	MSP-1	22	7347	ACAAGTGGAACAGCTGTTACAA
RO33	MSP-1	24	7918	GTTGTTGCAAAGCCTGCAGGTGCT
IC1	MSP-2	21	6930	GCAGAAGCATCTACCAGTACC
FC27	MSP-2	19	6059	CACCTTCACCACCCATCAC

The probes are named after the isolates from which the sequence data were originally obtained (Kimura *et al.*, 1990; Smythe *et al.*, 1990).

2.6.6.2. Labelling of oligonucleotide probes.

The protocol was essentially as described by the manufacturer, using the 3'-oligonucleolabelling kit (Amersham). The labelling reaction was catalysed by terminal deoxynucleotidyl transferase to introduce a tail of fluorescein-dUTP onto the 3'-end of an oligonucleotide. 100×10^{-12} moles of each oligonucleotide were labelled with fluorescein-11-dUTP. The oligonucleotide and fluorescein were incubated with terminal transferase for 60-90 minutes at 37°C. The labelled probes could be stored at -20°C or used immediately.

2.6.6.3. Hybridization and detection of the probes.

Hybridization buffer was prepared as recommended by the manufacturers. The hybridization temperature was adjusted for each probe individually. For MSP-1, hybridization temperatures were 74°C, 56°C and 68°C for the K1, MAD20 and RO33 probes respectively. For MSP-2 the temperatures were 60°C for IC1 and 56°C for FC27. Stringency was controlled by hybridization temperature and post-hybridization washes, which were carried out at the hybridization temperature. Hybridization was carried out for one hour, with a probe concentration of 5-10ng/ml. Blots were first washed with 1 x SSC, 0.1% (w/v) SDS at room temperature, followed by higher stringency washes with 0.1 x SSC, 0.1% (w/v) SDS for 30 minutes at the hybridization temperatures. The blots were then washed briefly (1 minute) in buffer 1 (0.15M NaCl, 0.1M Tris, pH7.5) and incubated with constant agitation at room temperature for 30 minutes in block buffer (0.5% w/v blocking agent in buffer 1). They were then rinsed briefly (1 minute) in buffer 1.

Hybridized probes were detected by a two stage process; first, the fluorescein hapten was developed by incubation with anti-fluorescein horseradish peroxidase conjugate, and second, bound peroxidase was detected using ECL detection reagents.

The blots were first incubated in anti-fluorescein antibody diluted 1:1000 v/v in buffer-2 (0.4M NaCl, 0.1M Tris, pH7.5) for 30 minutes with constant agitation at room temperature. They were then transferred to a clean container and rinsed four times with excess of buffer-2, each rinse being carried out at room temperature for 5 minutes. Equal volumes of detection solutions 1 and 2 (ECL kit) were mixed to give a sufficient volume to cover the blots. The buffer was drained from the blots and they were transferred to a glass plate with the DNA side facing up. The detection solution was then poured directly to the blots and incubated for 1 minute precisely. The excess detection solution was drained off, the blots were wrapped with Saranwrap and exposed to a film. Exposure time varied between probes from approximately 30 seconds to 2 minutes.

2.6.6.4. Stripping of the probes

The original probe was removed from the membrane before reprobing with another one. For successful removal of probes, the membranes were not allowed to dry during the hybridization and detection procedure. Membranes were incubated in 0.1xSSC, 1%SDS and boiled for 15 minutes with agitation. They were then rinsed in 5xSSC at room temperature for 5 minutes, before hybridization with another probe, or stored wrapped with Saranwrap.

Chapter 3. Genetic diversity of *Plasmodium falciparum* in Sudan.

3.1 Summary

This chapter describes the detailed characterization of *P. falciparum* isolates collected in Asar, a small village in the Eastern Region, and in Khartoum, the capital and main urban centre of Sudan. Polymorphisms of 15 genetically controlled characters were studied.

3.2 Subjects and methods used.

In Asar village, thirty *P. falciparum* isolates were collected from patients attending a small clinic between 24th October and 4th November 1989 (section 2.1.1). The patients included sibling pairs from 2 houses, providing isolates 117/89 and 122/89 from two brothers and 106/89 and 110/89 a brother and sister. All patients were treated with chloroquine immediately after blood was collected.

In Khartoum, infected blood samples were obtained from 24 patients with *P. falciparum* during December 1988 and January 1989, who attended the Outpatients Department of the Khartoum Teaching Hospital and the Diagnostic Laboratory of the Malaria Administration Department in Khartoum. The patients came from different areas of the city, including Khartoum North, Omdurman, Elgref and Elklakla (Figure 5). 5 ml venous blood samples were collected from each patient with their consent, and processed as described in section 2.3.1., The patients included adults and children.

Isolates were processed and stored in liquid nitrogen (section 2.3.1.) for subsequent transport to Edinburgh, where they were established in *in-vitro* cultures (section 2.3.4.).

Isolates from Khartoum were characterized for serotypes of three polymorphic antigens (section 2.5.4) and variants of proteins detected by 2D-PAGE (2.5.2). Isolates from Asar were additionally examined for variants of polymorphic enzymes (section 2.5.1).

3.3 Results

3.3.1 Diversity of *P. falciparum* isolates in Asar

Twenty-nine of the thirty *P. falciparum* isolates collected from Asar village were successfully grown in culture. They were characterized for 15 different genetically controlled characters. Considerable diversity of these characters was detected. At least two variants of each character were present among the parasites of the village. For example, the enzyme GPI was present as two forms, GPI-1 and GPI-2. Some characters were highly polymorphic compared to others; for example there were 8 distinguishable alleles of MSP-1, and 7 alleles of each of the 2D-PAGE proteins D and K. Each isolate had a different combination of the alleles of each studied character. Details of the characteristics of the isolates are given in table 5.

(i) Enzymes.

Two forms of the enzymes GPI and ADA and three of PEP were detected among parasites of the village. GPI-1, ADA-1 and PEP-1 were the most common forms. ADA-2, PEP-2 and PEP-3 were each found in only a single isolate.

(ii) 2D-PAGE proteins

The proteins detected by 2D-PAGE were found to be very diverse. Each isolate had a unique combination of the different alleles of each protein. Some isolates possessed more than one variant of a particular protein, and this is indicative of multiclonal infections (Figure 10). Six alleles of

Table 5.

Genetic characterisation of *Plasmodium falciparum* from Asar village, eastern Sudan. 2D-PAGE proteins are polymorphic parasite proteins detected by two-dimensional polyacrylamide gel electrophoresis (section 2.5.2); letters refer to proteins determined by different genetic loci; and numbers indicate allelic forms of each protein; - indicates that a protein could not be detected, ? indicates uncertain identity of the allele. GPI, ADA and PEP are enzymes detected by electrophoresis (section 2.5.1), numbers indicate different variants of each enzyme. MSP-1 and MSP-2 (section 2.5.4) are merozoite surface antigens; numbers indicate allelic forms detected by monoclonal antibodies; ? indicates uncertain identity of the allele. Exp-1 is a blood stage antigen; +/- or -/+ denoted a mixed infection, the first symbol indicating the predominant type.

Table 5.

ISOLATE	2D-PAGE PROTEINS									ENZYMES		ANTIGENS		
	C	D	F	G	I	K	L	N	P	GPI	PEP	MSP-1	MSP-2	Exp 1
101/89	2	4a+5	2	1	1	6+1	3+1	-	1	1	1	28+20	2	+/-
102/89	-	-	1	1	1	10	3	2	1	1	1	49	2	-
104/89	2	3	1	1	1	6	1	2	1	1	1	28+20	2+7	-/+
105/89	2	8	1	2	1	3	3	-	2	1	1	20	4	+
106/89	9	5	1	1+2	1	3+1	3	1	1	1	1	20+26?	2+8	+
107/89	3	2	2	1	1	-	1	1+2	1	1	1	20	2	-
108/89	2	3	1	1	1	3	3	-	1	1	1	49+26?	2	-
109/89	-	-	1+2	2	-	-	1	2	1	2	3	15	2	-
110/89	-	4a+5	2	1	1	9+10	3	2	1	1	1	20	2	-
111/89	10	3	2	1	1	3	1	-	1	2	1	50	2	-/+
112/89	10	5	2	1	1	1	3	-	1	1	1	28	2	-
113/89	-	9	1	1	1	3	3	1	1	1	1	20+28	2+7	+
114/89	-	6	1	1	2?	-	3	-	1	2	1	44	2	+
115/89	-	4a	2	1	1	-	1	2	1	2	1	32	2	-
116/89	-	3	2	1	1	3	1	2	1	1	2	20	2	-
117/89	-	3	1	1	1	6	3	2	1	1	1	50	2	-
118/89	3	-	2	1	1	-	1	1+2	1	1	1	28	2	-
119/89	-	3	1	1	1	-	2	2	1	1	1	20	4	-
120/89	1?	3	1	1	1	3	3	2	1	1	1	49	2	-
121/89	9	5+3	1	1	1	-	3	-	1	1	1	20+28	2+8	+
122/89	-	3	2	1+2	1	10	1	2	1	1	1	28	2	-
123/89	10	4a+5	2	1	1	6	1+3	-	1	1	1	28	2	+
124/89	3	2	2	1	1	3	3	1	1	1	1	49+20	2+7	+
125/89	6	4a+8	2	1	1	3	3	-	1	1	1	28+20	2+7	+
126/89	10	4a	1	1	1	11?	1+3	-	1	1	1	28+20	4	-
127/89	-	-	2	1	1	-	1	1	1	1	1	20	4	+
128/89	-	-	1	1	1	2+10	3+1	1+2	1	1	1	50	2	+/-
129/89	-	3	2	1	1	10	3	-	1	1	1	50	2	-
130/89	-	-	1+2	1	1	3	3	1	1	1	1	49+20	2	+

protein C, seven of protein D, seven of protein K and two each of proteins F, G,I, L, N and P were detected.

All possible combinations of the allelic variants of proteins N, L and F were detected (table 6).

Association between the variants of these proteins were examined by chi-square tests between each pair of loci. No evidence of linkage disequilibrium between any of the alleles at these .

.loci was found at a significance level of $P = 0.05$ (Table 6).

Previous work has established that protein P is the enzyme ADA (Fenton and Walliker,1990). Variants of ADA and of protein P were correlated as expected (Table 5).

Table 6 Association between alleles of proteins L, N and F in *P. falciparum* in Asar.

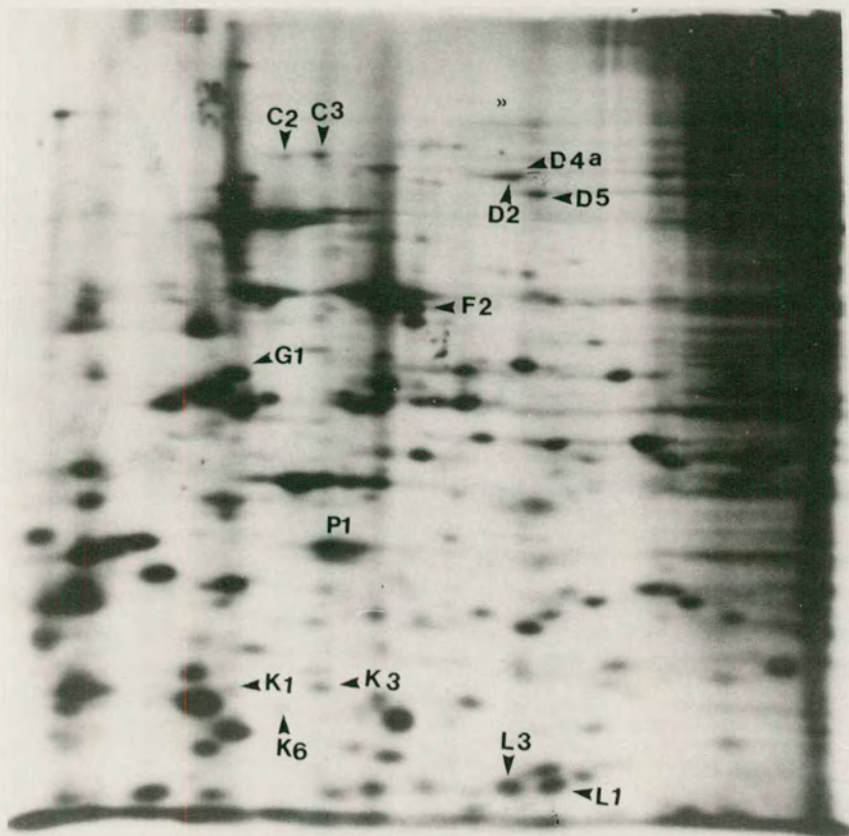
Protein	N-1	N-2	F-1	F-2
L-1	4 (4.0)	4 (3.5)	5 (7.5)	9 (6.8)
L-3	6 (5.0)	5 (5.0)	13 (10.8)	8 (10.0)
F-1	4 (3.6)	8 (6.5)		
F-2	4 (3.0)	6 (6.5)		

χ^2 tests were performed on the 3 data sets. Figures in brackets are the expected values on the basis of free association between alleles at each locus. The test for proteins L and F was significant at $P<0.01$. However, since 3 pairwise comparisons were performed, the conventional significance value ($P=0.05$) should be reduced by a factor of 3, i.e. to $P=0.017$, to eliminate spurious positive association; at this value, none of the associations were positive (significant).

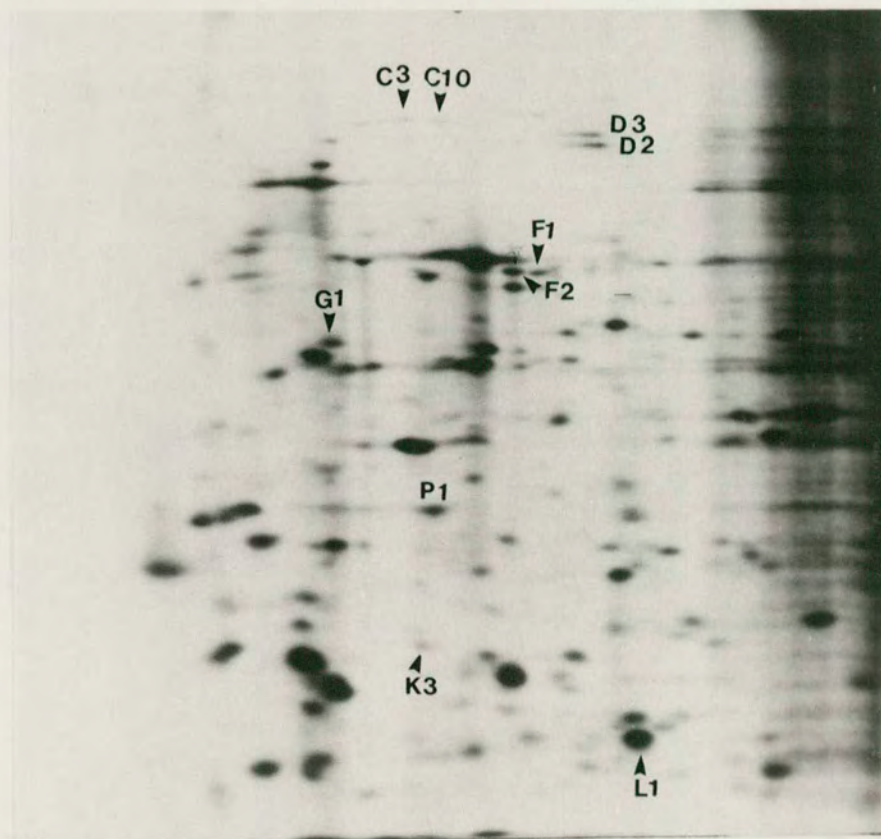
Figure 10.

Fluorograms of two isolates from Asar, 1989, (A) isolate 101/89 mixed with clone 3D7, the isolate shares some variants with 3D7 (F2, G1 & L1) and has its unique variants. Note the presence of more than one variant of two proteins in this isolate, D4a & D5, K1 & K6 indicating infection with more than one genotype. (B) isolate 111/89 mixed with 3D7 clone, 111/89 has its unique variants, D3, C10 & F1 and share some variants with 3D7, i.e. G1, P1, K3 & L1.

A Isolate 101/89 and clone 3D7



B Isolate 111/89 and clone 3D7



(iii) *Antigens*

Eight allelic serotypes of the MSP-1 gene recognized by specific Mab combinations believed to correspond to different alleles were detected in the village. Serotypes 49 and 50, recognized by Mab 6.1, were found in more than 40% of isolates. Mixed infections of parasites with different MSP-1 serotypes were identified in 10 patients.

Four allelic serotypes of MSP-2 were identified. With regard to the Exp-1 antigen, the variant recognized by Mab 5.1 was present in 45% of the isolates.

3.3.3. Diversity of isolates collected from patients of two households.

With regard to the sibling pairs, neither member of each pair was infected with identical genotypes (Table 7). Both members of pair 1 (isolates 106/89 & 110/89) showed a multiclonal infection with not less than 2 different clones. In pair 2 (isolates 117/89 & 122/89), one infection appeared to be of clonal type (117/89), but the other (122/89) had multiclonal infection distinguished by two forms of protein G. The two isolates infecting the two brothers were different. The parasites from each member within a household could be differentiated by a minimum of 5 different characters.

Table 7. Characteristics of *P. falciparum* of two household pairs in Asar

	C	D	F	2D-PAGE proteins				N	P	Antigens		
				G	I	K	L			M-1	M-2	Exp-1

Pair 1												
106/89	9	5	1	1+2	1	3+1	3	1	1	20+26	2+8	+
110/89	-	4a+5	2	1	1	9+10	3	2	1	20	2	-
Pair 2												
117/89	-	3	1	1	1	6	3	2	1	50	2	-
122/89	-	3	2	1+2	1	10	1	2	1	28	2	-

See table 5 legend, M-1(MSP-1) and M-2(MSP-2)

3.3.2 Diversity of *P. falciparum* isolates collected in Khartoum.

23 of the 24 isolates collected in the Khartoum area were established in culture. They were then examined for 2D PAGE proteins, and for serotypes of MSP-1, MSP-2 and Exp-1, using Mabs.

The isolates were found to be very diverse. Each could be identified by more than one variant of each character. Details are given in table 8.

Table 8. Characteristics of 23 *P. falciparum* isolates collected from Khartoum area.

Isolate	C	D	2D-PAGE proteins					L	N	P	Antigens		
			F	G	I	K	M-1				M-S	Exp-1	
301/88	9	2	2	1	1	8	3	-	1	20	2	-	
302/88	3	4	1	2	1	6	1	-	1	28	8	+	
303/88	3	5	2	1	1	8	3	-	1	50	8	+	
304/88	2	2	2	1	1	2+9	3	-	1	19	5+2	-	
305/88	-	4	2	1	1	-	3	-	1	20	10	+	
307/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	28	2+7	+	
308/88	2+3	6	2	1	1	1	3	-	1	ND	ND	+	
309/88	2	4	2	1	1	6	1	-	1	20+28	2+6	+	
310/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	28	2	+	
311/88	2+3	6	2	1	1	1	1	-	1	50	8	+	
312/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	50	2	-	
313/88	-	4+8	2	1	1	-	1	-	1	28	2+6	+	
315/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	1+52	8	+	
316/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	5+2	+	
317/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	6	+	
318/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	50	2	-	
319/88	-	4a	1	2	1	5	1	-	1	1+49	7	+	
320/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	50	7	+	
321/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	42	2	+	
322/88	10	4	1	2	1	-	3	-	1	20	5+10	+	
323/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	2	+	
324/88	-	4	2	1	1	-	3	-	1	27	2+7	+	
326/88	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	2	-	

ND= not done; see table 5 legend. M-1 (MSP-1) & M-2 (MSP-2)

(i) 2D-PAGE proteins

12 of the 23 isolates were examined for their 2D-PAGE proteins. As in Asar, considerable diversity was found. Each isolate had a unique combination of the different variants of each protein. Some isolates possessed more than one variant of a particular protein, which indicated multi-clonal infections, e.g. isolate 304/88 had two variants of protein K (K2 & K9). Six forms of protein C, seven of protein D, seven of protein K and two each of proteins F, G, I, N and P were detected.

(ii) Serotypes of MSP-1, MSP-2 and Exp-1 antigens

Nine allelic serotypes of MSP-1, recognized by specific Mab combinations, were detected among the 22 isolates. Serotypes 49 to 52 belonging to one of the two major alternative serological classes identified by Mabs such as 6.1 which recognise epitopes on domains 6 to 16 (Table 2 & Figure 3), were found in more than 30% of the isolates. The rest belonged to the second serological class recognised by a group of Mabs, e.g. Mab 9.2. Six allelic serotypes of MSP-2 were identified. The epitope on the Exp-1 antigen recognized by Mab 5.1 was present in more than 75% of the isolates.

3.3.6. Comparison between isolates in Khartoum and Asar.

(i) Multi-clonal infections

Since the blood forms of the malaria parasite are haploid, the occurrence of more than one variant of a given character in an isolate must mean the presence of more than one genotype (clone). In 15 out of 29 isolates from Asar and 11 out of 23 from Khartoum, more than one form of at least one character was detected. The precise genotypes present in each isolate could only be identified by cloning, but this was not done in the present work except in the drug-resistance

studies (chapter 5). Such multiclonal infections have been reported from other *P. falciparum*-endemic countries (e.g. Creasey *et al.*, 1990; Conway, Greenwood & McBride 1991).

(ii) MSP-1 serotypes

Figure 11 shows the frequencies of the MSP-1 alleles detected in Asar and Khartoum. Serotype 20 was the most frequent form in both areas, followed by serotypes 28 and 50, with the exception of serotype 49 in Khartoum. Serotypes 15, 26, 32 and 44, were found in Asar but not in Khartoum. On the other hand serotypes 1, 19, 27, 42 and 52 were found in Khartoum but not in Asar. In total, nine serotypes of the MSP-1 were detected among the 24 isolates from Khartoum area while only eight were found in the 29 isolates from Asar. This provides some limited evidence that the parasite in village, Asar, has a more restricted gene pool for these antigens compared to those of the urban area (Khartoum).

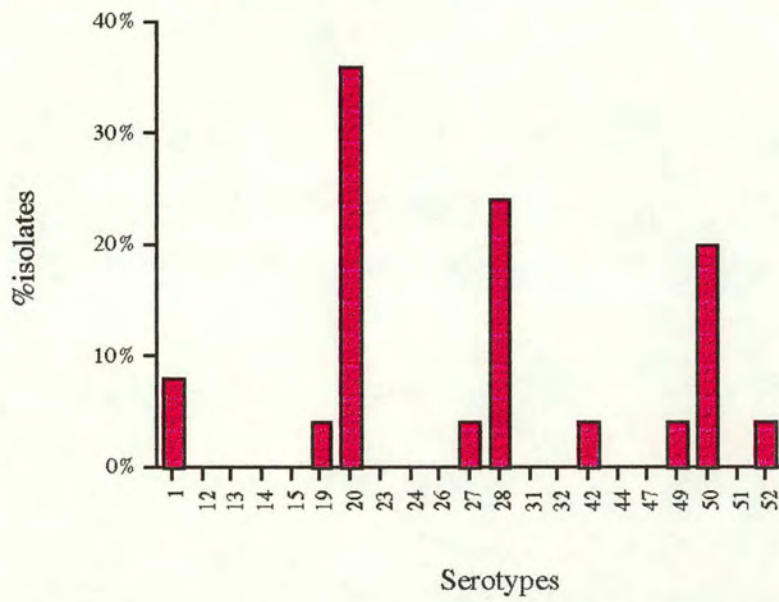
(iii) MSP-2 serotypes

Figure 12 represents the distribution of MSP-2 serotypes in Asar and Khartoum. Serotype 2 was the most frequent in both areas while serotype 4 was detected at low frequencies in the two places. Serotypes 6, 7, and 8 were detected only in Khartoum. Thus, as with the MSP-1 alleles, more MSP-2 alleles were detected in Khartoum (urban area) than in Asar (village).

Figure 11.

Allelic serotypes of MSP-1 in (A) Khartoum central, Sudan (urban area) and (B) Asar village, eastern Sudan. Each serotypes was defined by its unique reactivity with MAbs (section 2.5.4.); serotypes 20 & 28 are frequent in both areas, however, each area has unique serotypes which are lower frequencies, i.e. serotype 1, 19 & 52 in Khartoum and serotypes 15, 26, 32 & 44 in Asar.

(A) MSP-1 Serotypes Khartoum 1988



(B) MSP-1 serotypes Asar 1989

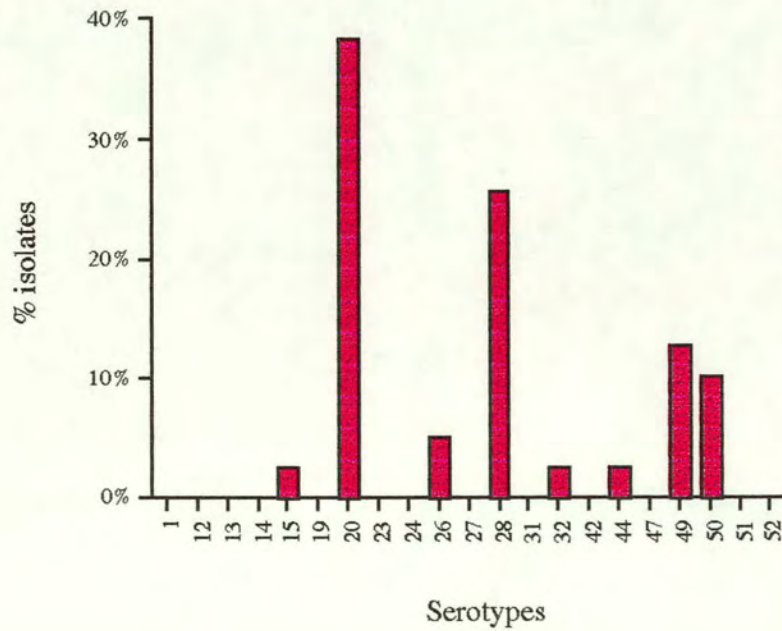
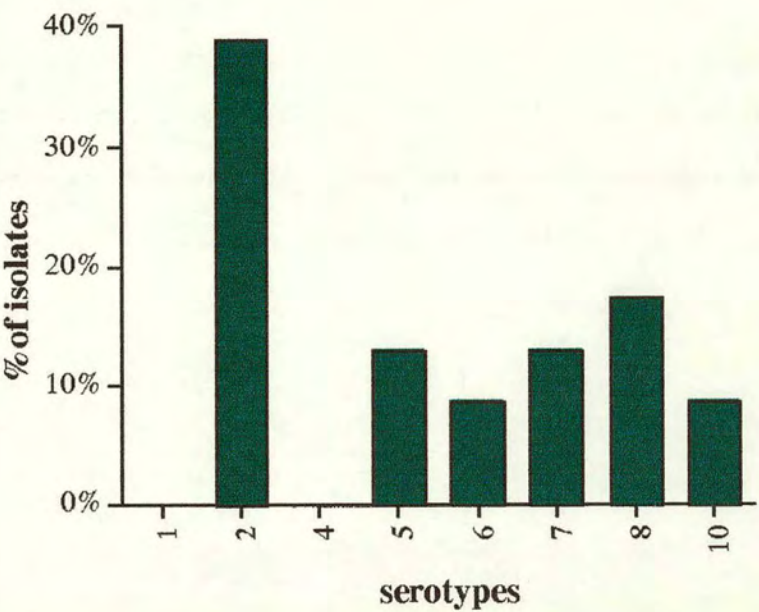


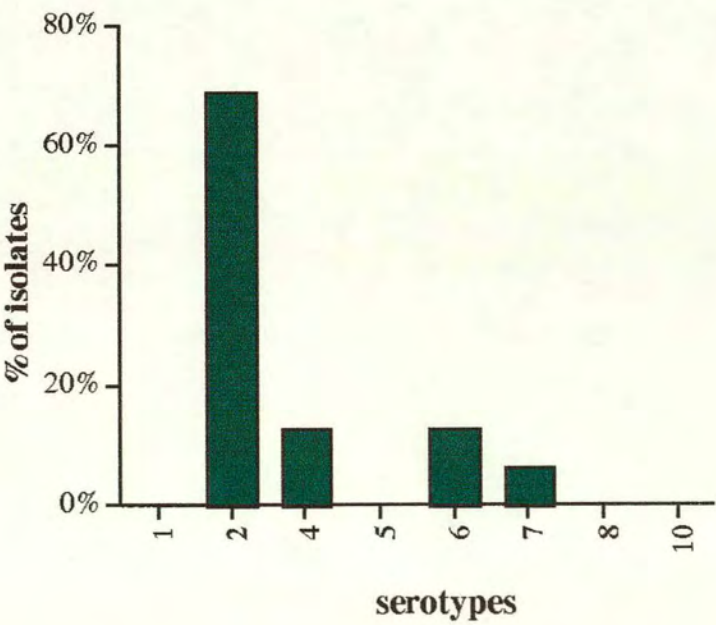
Figure 12.

Allelic serotypes of the MSP-2 in (A) Khartoum central Sudan, Urban area, and (B) Asar village eastern Sudan. Each serotypes was defined by a unique reactivity with MAbs (section 2.5.4.), serotype 2 is frequent in both areas, each area has infrequent unique serotype frequencies, i.e. serotype 5, 8 & 10 in Khartoum in 1988 and serotype 4 in Asar in 1898.

A. Khartoum



B. Asar



3.4. Discussion

Malaria is one of the most serious health problems in Sudan, constituting up to 40% of all infectious diseases (Sudan, 1986). The predominant parasite species is *P. falciparum*, which causes about 90% of all reported malaria cases (Omer, 1978). Until now, no information has been available on the genetic composition of the *P. falciparum* population in Sudan. Such information is needed for the planning of new control measures, especially in view of the increasing number of drug-resistant forms of this parasite (Bayoumi *et al.*, 1989; Ibrahim *et al.*, 1992; Awad-Elkariem *et al.*, 1992).

Previous studies on natural *P. falciparum* populations using the genetic markers studied here have established the following: (a) considerable diversity exists among these populations, (b) infection with more than one genotype is common and (c) geographic variations occur in frequencies of variants of these markers (Thaithong *et al.*, 1989; Carter & McGregor, 1973; Carter and Voller, 1975; Sanderson *et al.*, 1981; Creasey *et al.*, 1990). However, in none of these studies was systematic sampling of parasites carried out to examine the extent of polymorphism in space or time. Conway & McBride (1991) studied a large number of *P. falciparum* isolates for antigen diversity in an urban/periurban area in The Gambia over a two year period. They have shown that not less than 35 different alleles of the MSP-1 and 8 of MSP-2 co-exist in that area.

The most significant finding of this study was the remarkable degree of polymorphism that occurred in a small population (Asar village). At least 2 alleles of each of the genes investigated were found, and each of the 29 patients examined during a two week period contained parasites of different genotypes. This finding agrees with that of Creasey *et al.* (1990), who showed that no 2 isolates collected from 60 patients from different regions of Thailand, Zimbabwe and Brazil were identical.

The diverse forms of the characters examined here have been well documented in previous surveys of natural parasite populations. The electrophoretic forms of enzymes studied were described before from other African countries (Carter and Voller, 1975; Sanderson *et al.*, 1981; Creasey *et al.*, 1991). The frequencies of the forms of GPI and ADA found in Sudan accord with those reported in these earlier studies. The discovery of three forms of PEP in a single village is of interest. PEP-1 is by far the most common form of this enzyme in most countries, with the exception of Tanzania, where 6 of 8 isolates examined by Sanderson *et al.* (1981) were PEP-2 and 2 were PEP-3. The presence of forms 2 and 3 in Asar suggests that these forms are more common in East than in West Africa.

All forms of the 2D-PAGE proteins C,D,F,G, and P detected in Khartoum were found to occur in Asar, but with different frequencies. Some variants of protein K detected in Asar, K10 & K11, were not seen in Khartoum. Similarly some seen in Khartoum, K5, K8 & K9, were not seen in Asar. The variants of these proteins in Sudan occurred at similar frequencies to those seen in other countries, especially Zimbabwe (Creasey *et al.*, 1990). Forms of protein C were difficult to detect in many isolates from both Khartoum and Asar. Protein C10 is a new allele reported here for the first time, and was found among parasites from both study areas. Protein C2, present in 4 isolates in each area, had previously been found only in Thailand. Protein C3 and C9, detected in both areas have been found only in African countries. Considerable variation was also found in protein K, as seen elsewhere (Creasey *et al.*, 1990). K10 is a previously undescribed form of this protein.

The frequencies of the MSP-1 alleles were similar to those in Zimbabwe. Mab 6.1 determined the K1-type of dimorphic class of MSP-1, (alleles 49 to 52); parasites positive for this Mab are as frequent in Khartoum and Asar as in Zimbabwe, but occur at low frequency (less than 5%) in The Gambia & Nigeria (Creasey *et al.*, 1990; Conway *et al.*, 1992).

Multiclonal infections were detected in 7 out of 24 patients in Khartoum and 15 out of 29 patients in Asar. Similar observations of multi-clonal infections have been made by others (Carter

& McGregor, 1973; Thaithong *et al.*, 1984; Lockyer *et al.*, 1989; Creasey *et al.*, 1990; Conway Greenwood & McBride, 1991). The precise genotypes of such isolates can normally be identified only by cloning. However, it seems clear from the surveys of uncloned isolates that genetically identical *P. falciparum* clones are rarely found. The results from the two household pairs showed that even in circumstances where infection could have been contracted at the same time, the parasites in each patient were different. Such infections might have been initiated by 2 or more mosquitoes infected with genetically different sporozoites, or by different sporozoites from the same mosquito.

Comparison between parasites from Khartoum and Asar revealed that allelic diversity was slightly lower in the village (Asar) compared to the urban area (Khartoum). 9 serotypes of the MSP-1 and 6 of the MSP-2 were detected among 24 isolates from Khartoum, while 8 and 4 respectively were identified among 29 isolates in Asar village. Similar observations were reported from The Gambia. The 3-locus genotype diversity (MSP-1, MSP-2 and Exp-1) observed in two villages was lower than the expected value calculated for an identical sample size taken from an urban region (Conway & McBride, 1991). In Asar the parasite gene pool could be limited by the population size and the short transmission season. Moreover, random genetic drift in small populations can account for loss of rare alleles (Kimura & Crow, 1964).

The diversity of the parasite genotypes in Asar and Khartoum can be explained most satisfactorily as a result of crossing and recombination between different parasites. In a cross between 2 genetically distinct *P. falciparum* clones recombination had occurred between all the proteins and enzyme variants used in this study (Walliker *et al.*, 1987; Fenton & Walliker 1990). Pairwise comparisons between the 2D-PAGE proteins L-N, L-F, and N-F detected in Asar (table 4) showed that all the possible combinations of the different alleles of each gene pair occurred. While the number of isolates tested was small, there was no evidence of linkage disequilibrium (table 6); the alleles assorted independently, presumably due to random recombination during meiosis (chapter 6). It could be considered therefore that a non-recombining clonal population

structure for *P. falciparum* as that proposed by Tibayrenc *et al.* (1990; 1991), is not appropriate to describe the parasite population in this village. Recently, an independent statistical analysis of the Asar data also concluded that the diversity of parasite genotypes are consistent with frequent sexual recombination (Maynard Smith *et al.*, 1993).

Chapter 4 Seasonal variations in the *Plasmodium falciparum* population in Asar village.

4.1. Summary.

The frequencies of alleles of many polymorphic loci of *P. falciparum* parasites in Asar village were monitored over three consecutive years. The genes studied were those encoding characters including parasite surface antigens, proteins detected by 2D-PAGE and enzymes. Results are discussed in reference to the population dynamics and structure of *P. falciparum* in an area where malaria transmission is seasonal.

4.2. Subjects and methods used.

Isolates of *P. falciparum*.

Isolates were collected from Asar village (section 2.1.1.). Patients of all age groups who had pure *P. falciparum* infections were included. 32, 39 and 43 venous blood samples were collected, with informed consent, during the transmission seasons of 1989, 1990 and 1991 respectively. The isolates were processed (section 2.3.1.) and some of them were later cultured *in-vitro* (2.3.4.)

Methods used.

29 isolates from 1989 and 16 isolates from 1990 were characterised for the following characters: (1) Serotypes of three polymorphic antigens, MSP-1, MSP-2, and Exp-1, which were classified according to their reactivity with Mabs as described by Conway & McBride (1991) (section 2.5.4.). (2) Electrophoretic variants of the enzymes, GPI, ADA and PEP (section 2.5.1.). (3) Nine polymorphic proteins denoted C, D, F, G, I, K, L, N & P identified by 2D-PAGE (section 2.5.2.).

32, 39 & 43 isolates collected during 1989, 1990, & 1991 respectively were examined for alleles of MSP-1 and MSP-2 using PCR and sequence-specific oligonucleotide probes (section 2.6). The DNA samples extracted from these isolates (section 2.6.1. & 2.5.2.) were subjected to PCR using primers to amplify a polymorphic region in each gene (section 2.6.4.). Variations in the lengths of the amplified-regions were identified as size differences on agarose gels (section 2.6.4.2), while variations in the sequence of the amplified fragments were identified by hybridisation with sequence-specific probes (2.5.6).

4.3. Results

Extensive diversity was detected among the isolates collected in 1989 (chapter 3), 1990 and 1991. Each of the 29 isolates of 1989 (chapter 3, Table 5) and the 16 of 1990 (Table 9) had different combinations of the alleles of each of the characters studied. None of the isolates studied in 1990 contained the same combinations of alleles as any from the previous year.

More than one form of at least one character was detected in 15 of the 29 isolates from 1989 and in 19 of the 39 isolates from 1990. The 1991 isolates were studied for only two characters, however; three isolates were found to contain more than one clone.

4.3.1. Variants of enzymes, 2D-proteins, antigens serotypes and response to antimalarial drugs over two consecutive transmission seasons, 1989 and 1990.

The blood form of the parasite is haploid and thus whenever more than one variant of each character occurs, this indicates a multiclonal infection. Multiclonal genotypes occur in different proportions and it is technically difficult to determine the exact proportion of different genotypes in an isolate. Most of the techniques in use identify the variants qualitatively only. The Mabs with different isotype specificity can quantify multiclonal infections (Conway *et al.*, 1991).

Table 9. Genetic characteristics of *P. falciparum* isolates collected from Asar during 1990.

ISOLATE	C											GPI					ADA	PEP	MSP-1	MSP-2	Exp	1
	C	D	F	G	I	K	L	N	P													
101/90	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	1	1	1	1	1	1	1	1	1	1	1	-
102/90	-	-	-	-	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	-
103/90	3	4	-	-	1	1	-	2+1	1	1	1	1	1	1	1	1	1	1	1	1	1	-
104/90	-	-	1	2	1	6	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-
105/90	4	4	1+2	1	1	-	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	+
106/90	1	-	2	1	1	3	3	1+2	1	1	1	1	1	1	1	1	1	1	1	1	1	+
107/90	3	2	1+2	1	1	3	1	1+2	1	1	2	1	1	1	1	1	1	1	1	1	1	+
110/90	-	2	1	1	-	6	3	2	1	1	1	1	1	1	1	1	1	1	1	1	1	-
111/90	-	-	1	1	1	10+11	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-
114/90	3	1	2	1	1	3+11	1	2+1	2	2	2	2	2	2	2	2	2	2	2	2	2	-/+
115/90	-	2	2	2	1	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	-
116/90	6	2	1+2	2	1	3	1	2+1	1	1	1	1	1	1	1	1	1	1	1	1	1	-
117/90	-	4a	1	-	1	2+3	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	+
118/90	10	1	2	2	1	3	3	2+1	1	1	1	1	1	1	1	1	1	1	1	1	1	+
119/90	3	1	-	2	1	6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	+
120/90	3	1	1	1	1	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-
123/90	3	1	1	1	1	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-
124/90	3	2	1+2	1	1	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	-
127/90	nd	nd	nd	nd	nd	nd	nd	nd	nd	2	2	2	2	2	2	2	2	2	2	2	2	-
136/90	-	-	2	1	1	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-
139/90	4	2	2	1	1	3+9	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	+
140/90	nd	nd	nd	nd	nd	nd	nd	nd	1	1	1	1	1	1	1	1	1	1	1	1	1	-

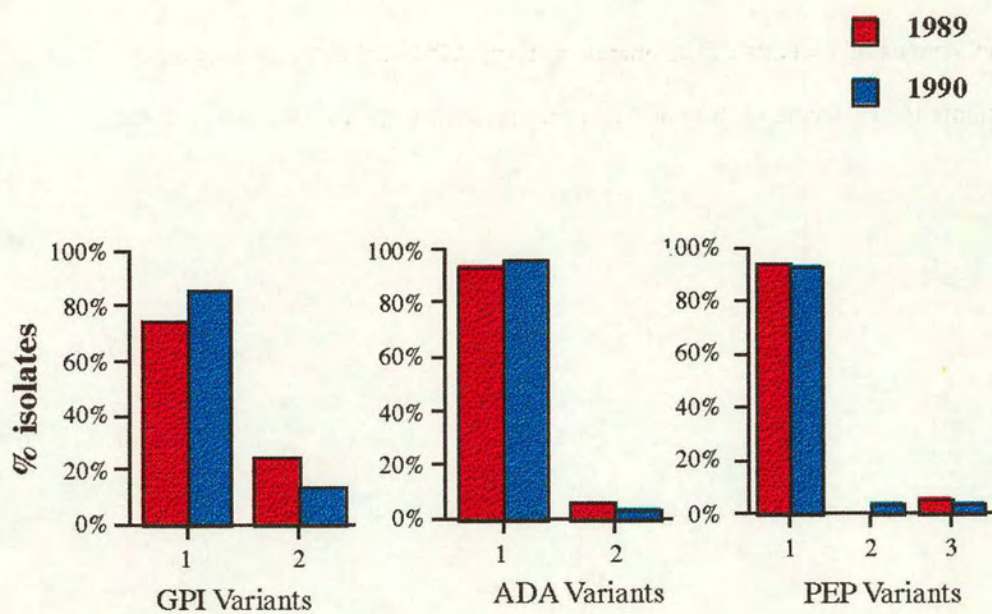
See table 5 legend. nd= not done

Figure 13.

Prevalences of variants of the enzymes, GPI, ADA & PEP among isolates collected from patients in Asar village, Sudan during transmission season of 1989 & 1990.

Figure 13.

Prevalences of variants of the enzymes, GPI, ADA & PEP among isolates collected from patients in Asar village, Sudan during transmission season of 1989 & 1990.



However, for the purpose of this study, it was assumed that variants of multiclonal infections occurred in equal proportions

Enzymes.

Two forms of GPI and ADA were identified in 1989 and 1990 in the village. GPI-1 was the most frequent allele in both years. The frequency of GPI-1 increased slightly in 1990, while GPI-2 decreased (Figure 13). The allele frequencies of both ADA and PEP enzymes remained more or less unchanged, with the exception of the rare variant PEP-2 which was detected only in 1989. GPI-1, ADA-1 and PEP-1 remained the most common alleles in both years (Figure13).

2D-PAGE proteins

The proteins detected by 2D-PAGE were found to be very diverse in each of the two transmission seasons. In both 1989 and 1990 each isolate was found to have a unique combination of variants of the different proteins.

There was a clear difference in the frequencies of the highly polymorphic proteins C, D and K, between isolates collected in 1989 and 1990. Some rare alleles in 1989 were not detected in 1990, for example proteins C1, C2, C 9, D3, D6, D8, D9, and K1, while others new alleles appeared in 1990 including C4, D1 and D4. The frequency of protein G variant G2 increased from 1989-1990, and a rare variant G3 occurred in one isolate in 1990.

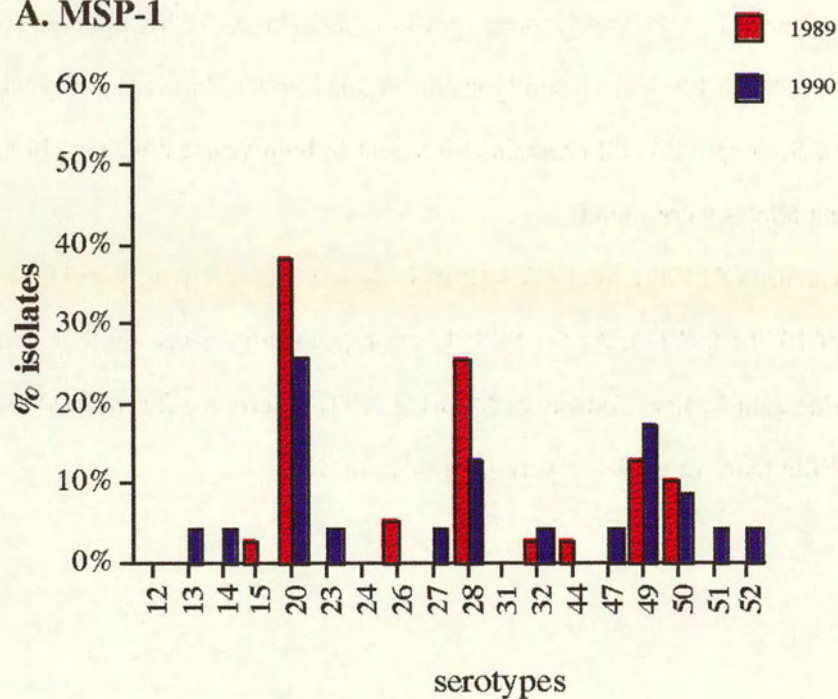
With regard to the other less polymorphic proteins, the frequency of the two variants of proteins F, N and P did not change over the two years. The frequency of variants of proteins L and I was also constant over the two studied years, with the exception of rare variants L2 and I2, each of which occurred in one isolate in 1989, but not at all in 1990.

Figure 14.

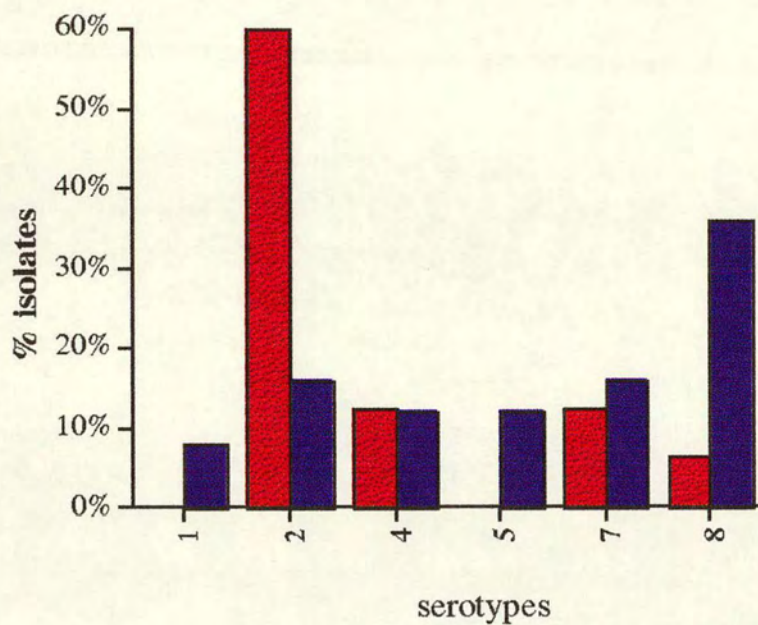
(A) Prevalence of MSP-1 allelic serotypes in Asar village, Sudan, during the transmission season of 1989 & 1990. Each serotype was defined by a unique reactivity with MAbs (see figure 9). Serotypes 20 & 28 remained frequent in both years; however, in each year some infrequent alleles were found.

(B) Prevalence of MSP-2 allelic serotypes in Asar village, Sudan, during the transmission season of 1989 and 1990. As for MSP-1, serotypes were defined by their unique reactivities with MAbs (Conway & McBride, 1991). Serotype 2 remained frequent in both years, while more rare alleles were seen in 1990.

A. MSP-1



B. MSP-2



Serotypes of the MSP-1, MSP-2 and Exp-1 antigens.

Eight and twelve serotypes of MSP-1 were detected in 1989 and 1990 respectively. Five out of the eight serotypes detected in 1989 were found in 1990 with comparable frequencies, while the other three less frequent 1989 serotypes 15, 26, and 44 were not present in 1990. Similarly, seven new serotypes, 13, 14, 23, 27, 47, 51 and 52, were detected in 1990. Serotypes 49 to 52 (Figure14a), were found in 31% and 36% of isolates in 1989 and 1990 respectively.

Four and six serotypes of MSP-2 were identified in 1989 and 1990 respectively. The most frequent serotype in 1989, serotype-2, reduced significantly in frequency in 1990 (from 69% to 16%). In contrast, serotype 8 which occurred at low frequency in 1989, increased in frequency in 1990. Two new two serotypes, 1 and 5, were detected at low frequencies in 1990 (Figure14b).

The Exp-1 antigen allele containing the epitope recognized by Mab 5.1 did not change markedly in frequency, from 45% in 1989 to 41% in 1990.

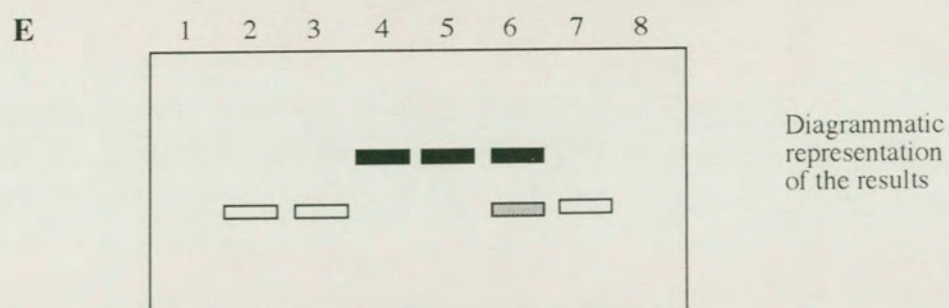
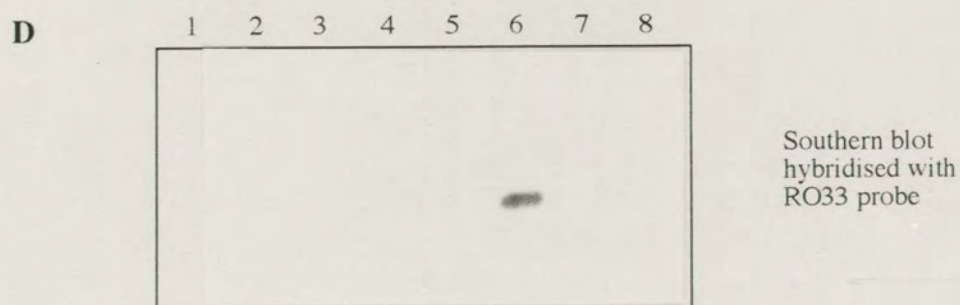
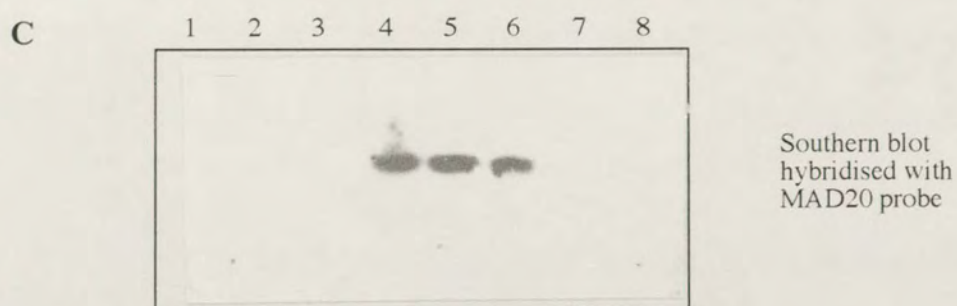
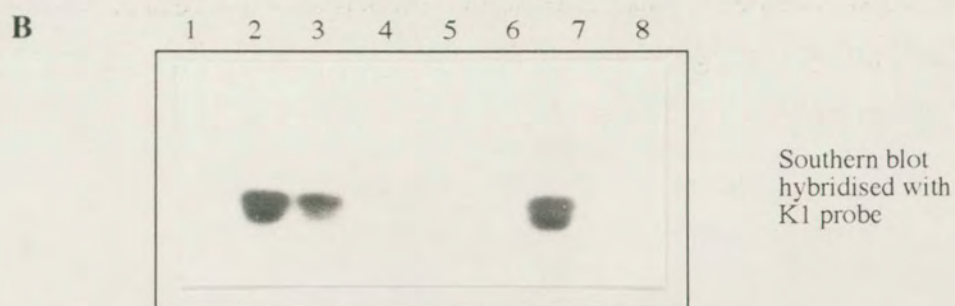
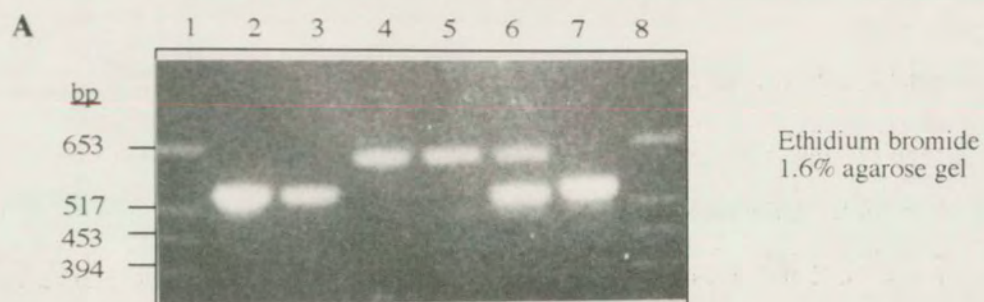
4.4.2. Comparison of alleles of polymorphic regions of MSP-1 and MSP-2 genes, between 1989-1991.

MSP-1 alleles.

31, 39 and 42 isolates were examined by PCR/DNA probes in 1989, 1990 and 1991 respectively. A very high degree of variation was observed between isolates from each transmission season. 6, 8 and 9 different alleles were identified within the village in 1989, 1990 and 1991 respectively, distinguishable by size and/or sequence (Figure15 & 16). The most frequent alleles varied in frequencies from one season to another. However, some alleles of K1-type (Figure16a), MAD20-type (Figure 16b) or RO33-type (Figure16C) were identified in 1990 and/or 1991 but not in 1989.

Figure 15.

Alleles of MSP-1 gene of *P.falciparum*, classified by PCR and sequence specific probes, in isolates from Asar. PCR-amplified fragments separated by electrophoresis (panel A), Southern blots of these fragments were hybridized with allele-specific probes K1, MAD20 and RO33 (panel B, C and D respectively). Tracks 1 and 8 are size markers, tracks 2-7 are *P.falciparum* isolates. Note size and sequence variation between isolates. Isolate in track 6 has two alleles differing by sequence and size. Panel E, shows a diagrammatic representation of the results.



Key:

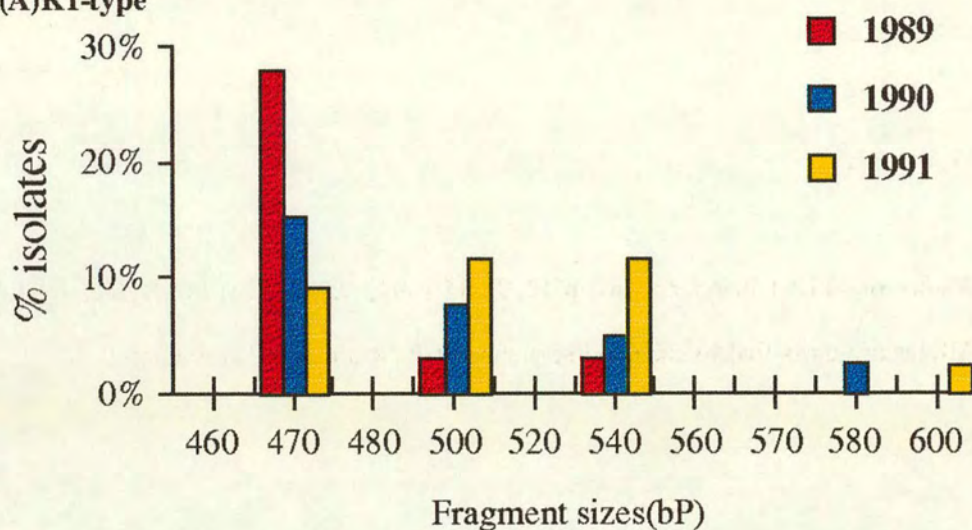
□ K1 ■ MAD20 ▒ RO33

Figure 16.

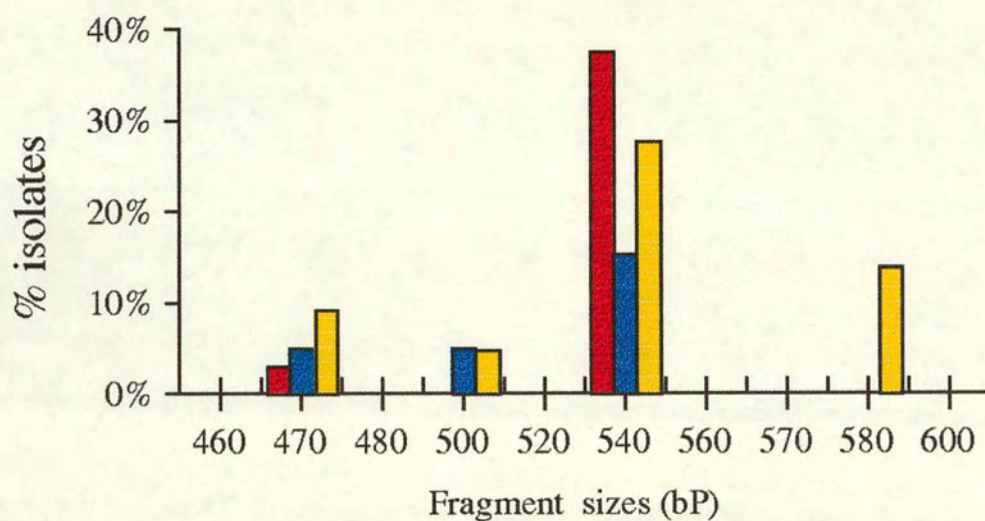
Alleles of MSP-1 in Asar between 1989 and 1991, (a) K1, (b) MAD20 & (c) RO33.

Alleles are classified by size and sequence of PCR-amplified fragments.

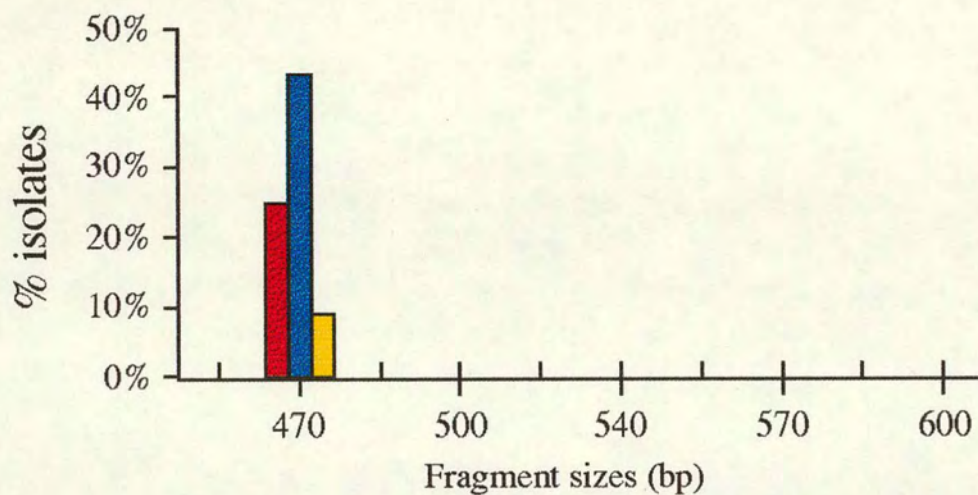
(A) K1-type



(B) MAD20-type



(C) RO33-type



Due to the small size of the sample, frequencies of alleles were sorted according to sequence only and were compared over the 3 years. All the known types (K1-type, MAD20-type and RO33-type) were detected throughout the study period. To examine whether variations in alleles occur from one year to another, a G-test was used, with the null hypothesis that allele frequencies remain stable from one year to another. There were no significant changes in frequency of K1 type over the studied period ($P > 0.8$), however, there was a highly significant change in the frequencies of MAD20-type and RO33-type alleles ($0.01 > P > 0.005$) (Table 10.). The frequencies of K1-type alleles were stable over the studied period ($G = 0.0613$ $P < 0.05$) (Table 11) compared to the MAD20 & RO33 alleles which varied significantly (Table 12 & 13).

Table. 10 Frequencies of MSP-1 alleles classified by K1, MAD20 & RO33 sequence, between 1989 and 1991 in Asar.

Year	K1-type Obs. (Exp.)	MAD20-type Obs. (Exp.)	RO33-type Obs. (Exp.)	Total
1989	10 (9.9643)	13 (13.01)	8 (8.026)	31
1990	12 (12.53)	10 (16.36)	17 (10.09)	39
1991	14 (13.5)	24 (17.62)	4 (10.87)	42
	36	47	29	112

$G=14.64$, $df=2$, $0.01 > P > 0.005$

Table 11 Frequencies of alleles of MSP-1 containing K1-type sequence between 1989 and 1991 in Asar.

Year	K1-type Obs. (Exp.)	not K1-type Obs. (Exp.)	Total
1989	10 (9.9643)	21 (21.0357)	31
1990	12 (12.53)	27 (26.4643)	39
1991	14 (13.5)	28 (28.5000)	42
	36	76	112

$G=0.061$, $df=2$,

Table. 12 Frequencies of MSP-1 alleles containing MAD20-type sequence between 1989 and 1991 in Asar.

Year	MAD20-type Obs. (Exp.)	not MAD20-type Obs. (Exp.)	Total
1989	13 (13.01)	18 (17.990)	31
1990	10 (16.36)	29 (22.633)	39
1991	24 (17.62)	18 (24.375)	42
	47	65	112

G=8.4272

Table 13 Frequencies of MSP-1 alleles containing RO33-type sequence between 1989 and 1991 in Asar.

Year	RO33-type Obs. (Exp.)	not RO33-type Obs. (Exp.)	Total
1989	8 (8.02680)	23 (22.9732)	31
1990	17 (10.0982)	22 (28.9018)	39
1991	4 (10.8750)	38 (31.1250)	42
	29	83	112

G=12.8696

MSP-2 alleles.

When alleles were sorted according to sequence and size, 7, 13 and 12 different alleles were identified within the village, in 1989, 1990 and 1991 respectively (Figure 17 & 18). The alleles identified over the 3 years were found to be comparable.

Figure 17.

MSP-2 alleles in *P.falciparum* isolates from Asar, classified by size and sequence. PCR fragments separated on 1.6% agarose gel stained with ethidium bromide. The fragments were blotted on to a nylon membrane and hybridized separately with the allele specific IC1 and FC27 probes. (B) Film exposed to blot hybridised with FC27 probe. (C) Film exposed to blot hybridised with IC1 probe. (D) Schematic representation of the results.

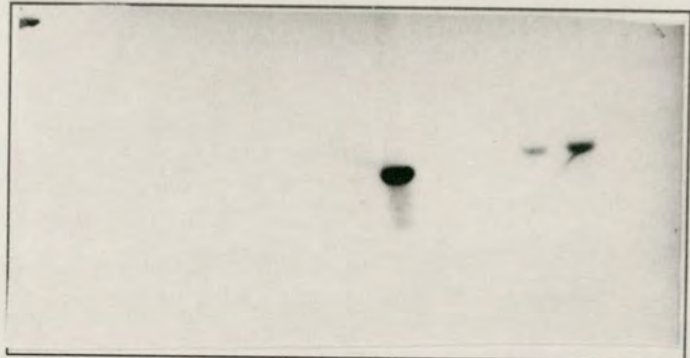
1 2 3 4 5 6 7 8 9 10 11

A



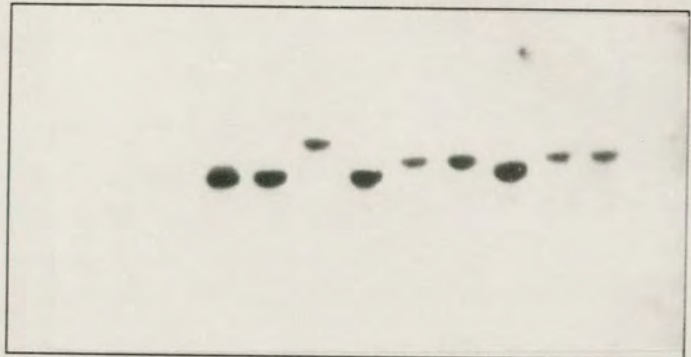
Ethidium bromide
1.6% agarose gel

B



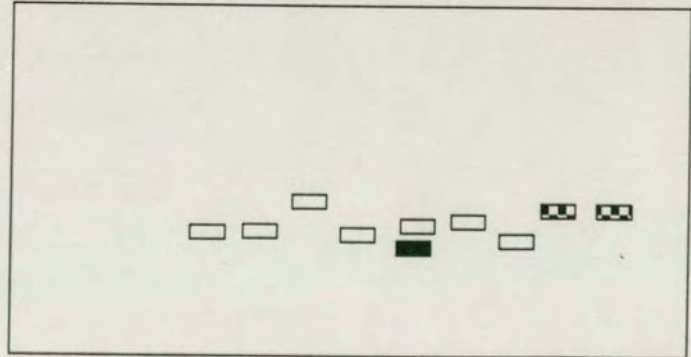
Southern blot
hybridised with
FC27 probe

C



Southern blot
hybridised with
IC1 probe

D



Diagrammatic
representation
of the results




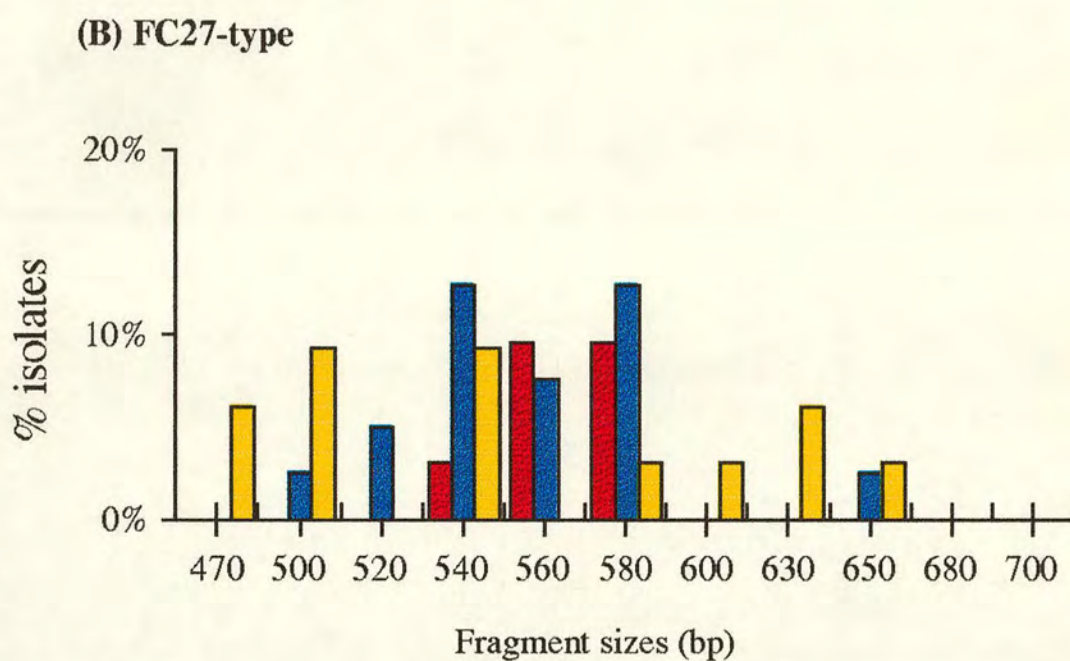
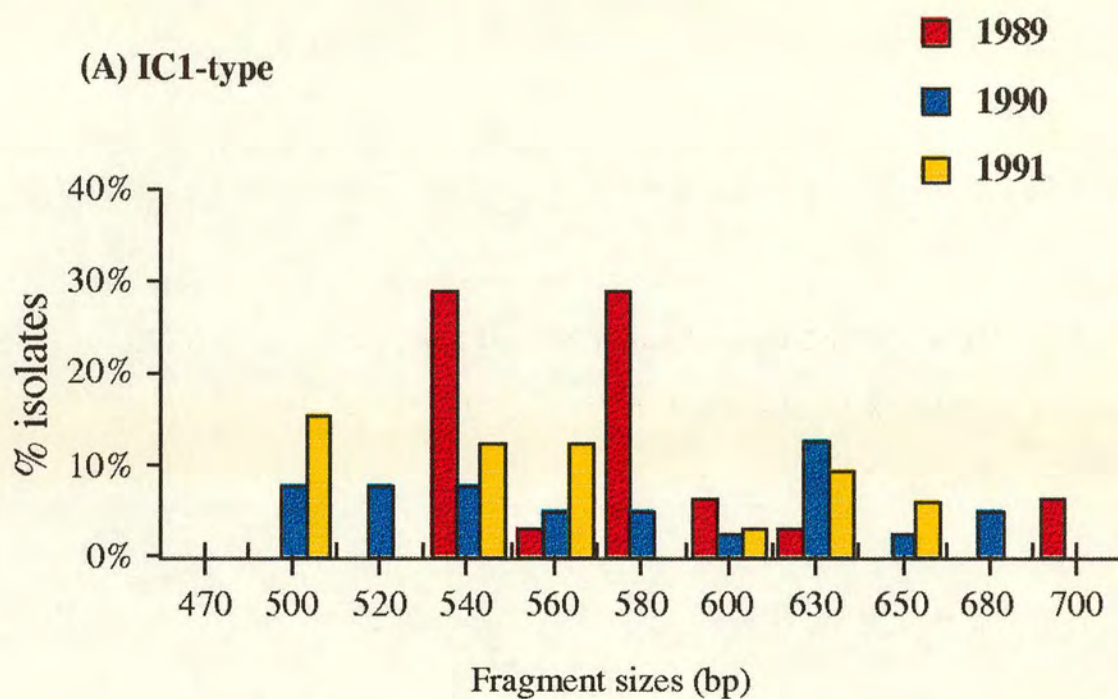
Key:  IC1  FC27
 FC27 and IC1

Figure 18.

Alleles of MSP-2 in Asar between 1989 and 1991, (a) IC1 & (b) FC27. Alleles are classified by size and sequence of amplified fragments.



As for MSP-1 the frequencies of the sequence variants, IC1 and FC27, were examined over the three studied years, using the G-test. However, the variation between 1989 and 1991, was not as significant as for MSP-1 (Table 14; $0.1 > P > 0.05$).

Table. 14. Frequencies of MSP-2 allele defined by IC1 & FC27-type sequence between 1989 and 1991 in Asar.

Year	IC1	FC27	Total
	Obs. (Exp.)	Obs. (Exp.)	
1989	24 (19.84)	7 (11.16)	31
1990	22 (25.60)	18 (14.40)	40
1991	23 (23.04)	13 (12.96)	36
	69	38	107

$G=5.4889$, $df=2$, $0.1 > P > 0.05$

4.5. Discussion

In semi-arid areas of central Africa the malaria transmission season is short and confined to the rainy months, becoming shorter as one moves away from the equator, depending on the level and duration of rains. At the edge of the Sahara desert the transmission season becomes very short and variable. In these areas transmission may cease and may retract to limited foci where scanty breeding sites persist during dry months of the year (Omer and Cloudsley-Thompson, 1970). Eastern Sudan is an example of such an area, where transmission occurs as a brief annual epidemic following rains, from September to November (Babiker *et al.*, 1991a).

A cross-sectional survey of the *P. falciparum* population in Asar in 1989 revealed a high level of genetic diversity (chapter 3). In this chapter allele frequencies of those genes within the village were monitored over three consecutive years, in an attempt to investigate whether the parasite population underwent any changes from one transmission season to another.

It is difficult to calculate allele frequencies in a given community from the parasite in blood samples. The parasite load varies from one patient to another, and multiclonal infections commonly occur with different proportions of clones in each patient. For the purpose of this study, an assumption is made that in a multiclonal infections the different genotypes exist at equal proportions. The frequency of a given allele was thus calculated based on the occurrence of that allele in an individual patient.

The variations in allele frequencies seen from one year to another in this study could possibly be explained by one or more of the following:

(i) The records of the village clinic indicate a sharp reduction of clinical malaria during the 9 months' dry season. The parasite population may well be subjected to a severe reduction in size during this period. A population "bottle-neck" at this time could be accompanied by random genetic drift (Hartle, 1987).

(ii) The parasite population in this village is not completely isolated as there is population movement within villages in the area and between the villages and Gedaref town (the main urban centre). Thus, population movement might be accompanied by introduction of new *P. falciparum* alleles which had not been detected in previous years.

(iii) Theoretically, fluctuations could result from immune-dependent selection. As the village residents become immune to particular alleles of a given antigen, the frequency of these alleles will decrease (Day *et al.*, 1992). Some of the antigens studied here (eg. MSP-1) may stimulate protective immune responses (Fruh *et al.*, 1991). However, little is known about the protective nature of these antigens and about the half life of any protective response. It is not known whether allele specific protection could be maintained to protect against subsequent challenge with the same allele the following year.

(iv) Less common alleles might not have been detected because of the small number of samples collected each season

In areas such as The Gambia where annual rainfall and transmission rates are higher than in the eastern region of Sudan, alleles of some of the genes studied here were found to be stable over time (Conway *et al.*, 1992). In the Gambia the parasite rate reduces slightly with the reduction in rainfall, and peaks during the rainy season, May to October (Greenwood *et al.*, 1987). Thus, the seasonal fluctuation in parasite prevalence may not be significant enough to affect gene frequencies and the parasite gene pool. In a randomly mating population, allele frequencies are expected to remain stable from generation to generation under stable environmental conditions and in the absence of selection. However, this stability may be affected by migration and mutation.

The stable frequencies of certain genes (i.e. enzyme variants GPI-1, ADA-1, PEP-1) may be due to the fact that these are less polymorphic loci, and that only two or three alleles of each exist in the village. However, this might also suggest that village-based parasites survive the dry season, and undergo resurgence following the annual rainfall and recolonisation of mosquito population. It has been reported that *A. arabiensis* can survive the long dry season of central Sudan as adults with some feeding activity (Omer and Cloudsley-Thompson, 1970). This implies that transmission might continue at a very low level during the dry season which would maintain the parasite population in the village. It has been reported that *P. falciparum* can last more than two years as a prolonged infection, in a semi-immune patient (Krajden *et al.*, 1991). However, there is no clinical or parasitological data from the village to support this hypothesis.

There is no information on the allele frequencies of the genes examined here in other villages in the area. Exophilic behaviour of *A. arabiensis* was reported in the Khashm Elgirba area, eastern Sudan, where mosquitoes were found resting in large numbers in natural outdoor shelter (Haridi, 1972). This could be a factor which leads to the dispersal of *P. falciparum* within villages in the area. Thus, the allele frequencies in Asar may reflect those of a much larger interbreeding population. Genetic similarities have been observed between geographically related parasites (Creasey *et al.*, 1990).

The observed fluctuations in allele frequency from one year to another in Asar village could probably be attributed to random genetic drift following the severe reduction in population size during the dry season. Nevertheless, the extensive diversity of the parasite genotypes within the village over the studied period suggests that the annual resurgence of the parasite population following the rains is accompanied by frequent sexual recombination between the genotypes that survive the dry season.

Chapter 5. Diversity of drug response in *Plasmodium falciparum* in Asar village, Sudan.

5.1. Summary

This chapter describes heterogeneity in response to, chloroquine, pyrimethamine and mefloquine in the parasite population in Asar. Responses to chloroquine and pyrimethamine were monitored over transmission seasons in two consecutive years, 1989 and 1990. This chapter also includes the development and use of a genetic method to distinguish R1-type chloroquine resistant forms from newly reinfecting parasites.

5.2. Introduction

15 years ago, *Plasmodium falciparum* in Sudan was reported to be sensitive to chloroquine (Omer, 1978; Carlin *et al.*, 1984). Chloroquine resistance was first suspected in a village north of Khartoum in the early 1980s (Altawil and Akood, 1983). However, there were no subsequent reports or failure of the standard therapy, 25mg/kg body weight over three days. As in other tropical countries there was a delay before chloroquine resistance appeared to be established. During 1985-1986 clinicians in eastern Sudan reported failure of the standard chloroquine therapy. Both *in vivo* and *in vitro* tests revealed that chloroquine resistance had become firmly established in this region (Bayoumi *et al.*, 1989). This was further confirmed by various other workers (Lienhard *et al.*, 1989; Saeed *et al.*, 1990; Babiker *et al.*, 1991b).

Chloroquine resistance in *P. falciparum* is defined as RI, RII or RIII, depending on the clinical response of patients to the standard dose of treatment (Bruce-Chwatt, 1986). In R1 resistance, parasites are temporarily cleared from the blood following chloroquine treatment, but re-appear several days after the treatment is terminated. These parasites are usually considered to represent a recrudescence of the initial parasites which are resistant. Alternatively, they could be due to re-infection of the patient with fresh parasites.

The alternative regimen for treatment of *P. falciparum* resistant to chloroquine is quinine and/or sulphadoxine/ pyrimethamine (Fansidar) (Bruce-Chwatt, 1986). Recently *P. falciparum* resistant to sulphadoxine/pyrimethamine (Fansidar) was reported from central Sudan (Ibrahim *et al.*, 1991)

5.3 Subjects and methods used.

Blood smears were taken from patients attending a daily clinic in Asar throughout the transmission seasons of 1989 and 1990. On diagnosis of *P. falciparum*, venous blood samples were immediately taken from each patient (section 2.2.2.), with their consent, and cryo-preserved in liquid nitrogen (section 2.3.1.). 30 isolates were collected in 1989 and 43 isolates in 1990. Each patient was then treated with a standard dose of chloroquine of 25mg/kg over three days. They were then examined daily for clearance of parasitaemia, which occurred by day 7 or earlier if the parasites were sensitive to the drug. Six patients, 2 in 1989 and 4 in 1990, returned to the clinic with febrile symptoms after periods ranging from ten to thirty days, all of whom proved to have *P. falciparum* in their blood. A further sample of blood was taken and cryo-preserved (section 2.3.1.). The patients were then treated with Fansidar (sulphadoxine/pyrimethamine), to take account of the possibility that the parasites could be resistant to chloroquine.

28 of the 29 isolates of 1989 and 16 of 1990 were established in *in vitro* culture (section 2.3.4.) for drug tests. When they had attained a satisfactory growth, their responses to chloroquine, pyrimethamine and mefloquine were tested *in vitro*. The minimum inhibitory concentration (MIC) of each drug which killed all, or nearly all, of the asexual forms of an isolate was determined (section 2.5.3.).

Clones were made (section 2.4) from some isolates which were known to be multiclonal when characterized for polymorphic markers (chapter 3), and their *in vitro* responses to chloroquine, pyrimethamine and mefloquine were tested (section 2.5.3.).

The parasites which caused the primary and secondary infections in the six patients suspected to have R1 type chloroquine resistance were typed for alleles of two polymorphic antigen genes, MSP-1 (section 1.6.3.a.) and MSP-2 (section 1.6.3.b.), using PCR/allele-specific probes (2.6.).

5.4.4 Results

5.4.1 Responses to chloroquine, pyrimethamine and mefloquine of the 1989 isolates and clones.

(i) The isolates

28 isolates were successfully tested for their responses to chloroquine, pyrimethamine and mefloquine. There was considerable variation in response to chloroquine and pyrimethamine, but less to mefloquine.

The MIC values of chloroquine for most of the isolates were high, similar to the resistant control clone Dd2; only 2 isolates had similar or lower MICs than the sensitive control clone 3D7. The remaining isolates had an intermediate response, with MICs which fell between these extremes.

There appeared to be 2 levels of response to pyrimethamine. Ten isolates had an MIC of 10^{-5} M or higher, a level similar to the resistant control clone HB3. The remaining 18 isolates were killed at concentrations of 10^{-7} M or less, similar to the sensitive control clone 3D7.

All the isolates appeared to be sensitive to mefloquine, being killed at a concentration of 0.4×10^{-6} M or less (Table 15).

There was no correlation between response to chloroquine and pyrimethamine, since all the possible combinations of resistance and sensitivity to the two drugs were detected among the isolates. For example, isolate 110/89 was resistant to both drugs, isolate 107/89 was sensitive to

both drugs, isolate 112/89 was resistant to chloroquine and sensitive to pyrimethamine and isolate 113/89 was resistant to pyrimethamine and sensitive to chloroquine. Though all the isolates were found to be sensitive to mefloquine, there was no association between that and responses to chloroquine and pyrimethamine.

Table 15. *In vitro* drug responses of 28 *P. falciparum* isolates from Asar village collected in 1989. Numbers indicate the minimum inhibitory concentrations (MIC) of the drugs which killed all or nearly all the parasites.

isolate	Chloroquine (MX10 ⁻⁶)	Pyrimethamine (M)	Mefloquine (MX10 ⁻⁶)
101/89	0.8	10 ⁻⁸	0.2
102/89	1.6	10 ⁻⁷	0.2
104/89	0.4	10 ⁻⁸	0.2
105/89	0.8	10 ⁻⁷	0.1
106/89	0.8	10 ⁻⁴	0.2
107/89	0.4	10 ⁻⁷	0.1
108/89	0.8	10 ⁻⁸	0.2
109/89	0.1	10 ⁻⁸	0.4
110/89	1.6	10 ⁻⁴	0.1
111/89	0.8	10 ⁻⁴	0.2
112/89	1.6	10 ⁻⁷	0.1
113/89	0.4	10 ⁻⁵	0.1
114/89	0.4	10 ⁻⁵	0.2
115/89	0.2	10 ⁻⁸	0.4
116/89	0.8	10 ⁻⁸	0.2
117/89	0.8	10 ⁻⁷	0.2
119/89	1.6	10 ⁻⁸	0.2
120/89	0.4	10 ⁻⁷	0.1
121/89	1.6	10 ⁻⁵	0.2
122/89	1.6	10 ⁻⁷	0.1
123/89	0.8	10 ⁻⁵	0.1
124/89	1.6	10 ⁻⁵	0.2
125/89	1.6	10 ⁻⁵	0.1
126/89	0.8	10 ⁻⁸	0.2
127/89	0.8	10 ⁻⁸	0.1
128/89	0.4	10 ⁻⁸	0.4
129/89	1.6	10 ⁻⁸	0.2
130/89	0.8	10 ⁻⁵	0.1
<u>Controls</u>			
Dd2 clone	1.6	10 ⁻⁵	1.6
HB3 clone	0.2	10 ⁻⁵	NT
3D7 clone	0.2	10 ⁻⁷	0.1

(ii) The clones

The work described in this section was done in collaboration with Professor Riad Bayoumi and colleagues. All clones were confirmed as pure by having a single allele of MSP-1 and MSP-2 using Mabs, as described in section 2.5.4 (Bayoumi *et al.*, 1993).

The responses of 20 clones to chloroquine, pyrimethamine and mefloquine are presented in table 10. Clones obtained from isolate 105/89 appeared to have intermediate response to chloroquine, resembling that of the original isolate (0.8×10^{-6} M). Isolate 106/89 contained clones with different levels of response to chloroquine, 0.2×10^{-6} M and 0.4×10^{-6} M, while the original isolate had a response of 8×10^{-6} M. The 3 clones of isolate 128 had an MIC (0.8×10^{-6} M) which was slightly higher than the original isolate (0.4×10^{-6} M). One clone each of isolates 102, 111/89, 122/89, 123/89 and 126/89 as well as three clones of 124/89, were all found to have a high MIC similar to the resistant control Dd2 and to their original isolates.

In agreement with the results of their original isolates, most of the clones were sensitive to mefloquine, except clone 123/5 which showed an intermediate response, higher than its original isolate (Table 16). Again in accordance with the response of the original isolates clones SUD 124/1, SUD 124/5, SUD 124/8, SUD 123/4 and SUD 111/1 were resistant to pyrimethamine. However, some variations were observed between clones and their original isolates. For example the clone series of 106 had a lower response than the 106/89 isolate.

Table 16. *In vitro* drug responses of 20 *P. falciparum* clones derived from isolates collected in 1989 from Asar village. Numbers indicate the minimum inhibitory concentrations (MIC) of the drugs.

isolate	Chloroquine ($\text{MX}10^{-6}$)	Pyrimethamine (M)	Mefloquine ($\text{MX}10^{-6}$)
SUD102/89/1	1.6	10^{-8}	0.2
SUD105/89/1	0.8	10^{-7}	0.4
SUD105/89/7	1.8	10^{-7}	0.2
1SUD05/89/9	0.8	10^{-7}	0.4
SUD105/89/11	0.8	10^{-7}	0.4
SUD106/89/1	0.2	10^{-6}	0.4
SUD106/89/7	0.4	10^{-6}	0.4
SUD106/89/9	0.4	10^{-6}	0.4
SUD106/89/10	0.2	10^{-6}	0.4
SUD106/89/11	0.4	10^{-6}	0.4
SUD111/89/1	1.6	10^{-5}	0.2
SUD122/89/1	1.6	10^{-6}	0.2
SUD123/89/5	1.6	10^{-5}	0.8
SUD124/89/1	1.6	10^{-5}	0.2
SUD124/89/5	1.6	10^{-5}	0.2
SUD124/89/8	1.6	10^{-5}	0.2
SUD126/89/1	1.6	10^{-7}	0.2
SUD128/89/1	0.8	10^{-8}	0.4
SUD128/89/4	0.8	10^{-8}	0.2
SUD128/89/5	0.8	10^{-8}	0.4
Controls			
Dd2 clone	1.6	10^{-6}	1.6
3D7 clone	0.4	10^{-8}	0.4

5.4.2. Comparison of responses to chloroquine & pyrimethamine in 1989 and 1990.

In 1990, 19 isolates were tested for their responses to both chloroquine and pyrimethamine. Their responses were compared to those of isolates collected in 1989 (Table 17).

The prevalence of parasites with a high MIC of chloroquine ($1.6 \times 10^{-6}\text{M}$) increased in the village in 1990. Even within the "sensitive" category ($\text{MIC} = 0.1 \times 10^{-6}\text{M}$; $0.2 \times 10^{-6}\text{M}$) susceptibility had slightly decreased. However, the prevalence of isolates that grew in levels of chloroquine considered to mark resistance (at or above $0.8 \times 10^{-6}\text{M}$) remained unchanged.

In contrast, susceptibility to pyrimethamine had increased in 1990 compared to 1989. 10 out of 28 isolates (37%) tested in 1989 had an MIC of 10^{-5} M or higher, a level similar to that of the resistant control HB3, compared to only 3 (16%) out of 19 in 1990. The remaining isolates in both years were killed at concentrations of 10^{-7} M or less (Table 17).

Table 17. Chloroquine and pyrimethamine susceptibility (MIC) of *P. falciparum* isolates from Asar village, 1989 and 1990.

Chloroquine ($\times 10^{-6}$ M)	isolates		Pyrimethamine (Molar)	isolates	
	1989	1990		1989	1990
1, 6 (Dd2)	9 (32%)	10 (53%)	10^{-4}	3 (11%)	0 (0%)
0.8	11 (39%)	3 (16%)	10^{-5} (HB3)	7 (25%)	3 (16%)
0.4	6 (21%)	6 (31%)	10^{-6}	0 (0%)	0 (0%)
0.2 (3D7)	1 (4%)	0 (0%)	10^{-7}	7 (25%)	4 (21%)
0.1	1 (4%)	0 (0%)	10^{-8} (3D7)	11 (39%)	12 (63%)
Total	28	19		28	19

5.4.3. Genetic evidence for R1 type chloroquine resistant *P. falciparum*.

Six patients, two in 1989 and four in 1990, did not respond to the standard dose of chloroquine, and showed possible recrudescent parasites following drug treatment. They thus exhibited clinical R1 type chloroquine resistance. Recrudescent and primary isolates collected from the six patients were typed for alleles of MSP-1 and MSP-2, using PCR combined with the use of non-radiolabelled gene probes (section 2.6.).

5.4.3.1. *In vivo* tests.

All the six patients who exhibited recrudescences showed an R1 type of chloroquine resistance response. In two patients, parasitaemias were cleared, but reappeared within seven days. The other four patients showed later recrudescences, two to three weeks after completion of treatment.

5.4.3.2. *In vitro* chloroquine response of the isolates which caused the primary infections.

The chloroquine response of the parasites of the primary infections of each of the six patients was tested *in vitro* (Table 18). The MICs varied from $0.4 \times 10^{-6}\text{M}$ to $1.6 \times 10^{-6}\text{M}$, compared to $0.2 \times 10^{-6}\text{M}$ for a drug-sensitive control clone 3D7.

Table 18. *In vitro* chloroquine responses of parasites of initial infections from six patients suspected to have R1 type of chloroquine resistance, taken before commencement of drug-treatment. 3D7: chloroquine-sensitive control clone. Dd2: chloroquine-resistant control clone. MIC: minimum inhibitory concentration of chloroquine.

Patient	MIC ($\times 10^{-6}\text{M}$)
107/89	0.4
123/89	0.8
101/90	0.8
103/90	1.6
120/90	1.6
124/90	1.6
Controls	
3D7 (Sensitive)	0.2
Dd2 (resistant)	1.6

5.4.3.3 MSP-1 and MSP-2 alleles of primary and recrudescence parasites.

The MSP-1 and -2 alleles of the parasites from these patients are shown in Table 19. Figure 19 illustrates the MSP-2 alleles of primary and recrudescence isolates of three patients. Each patient

was found to contain parasites with different combinations of alleles of the two genes. The recrudescence parasites in each patient possessed alleles identical to those of the parasites of the primary infections.

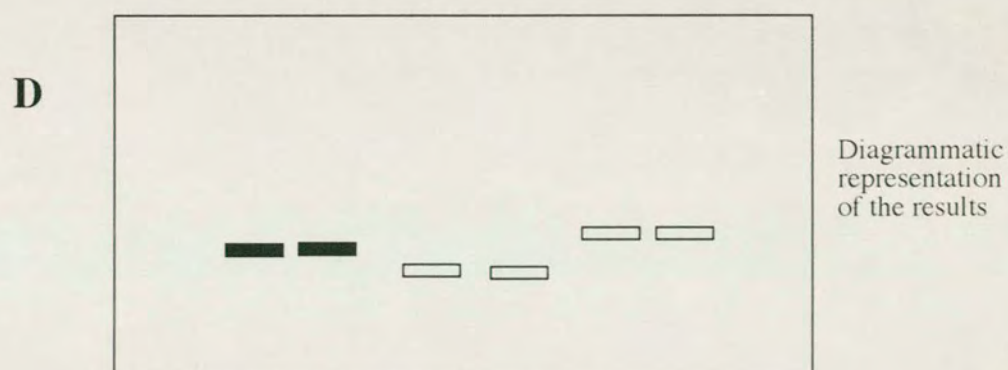
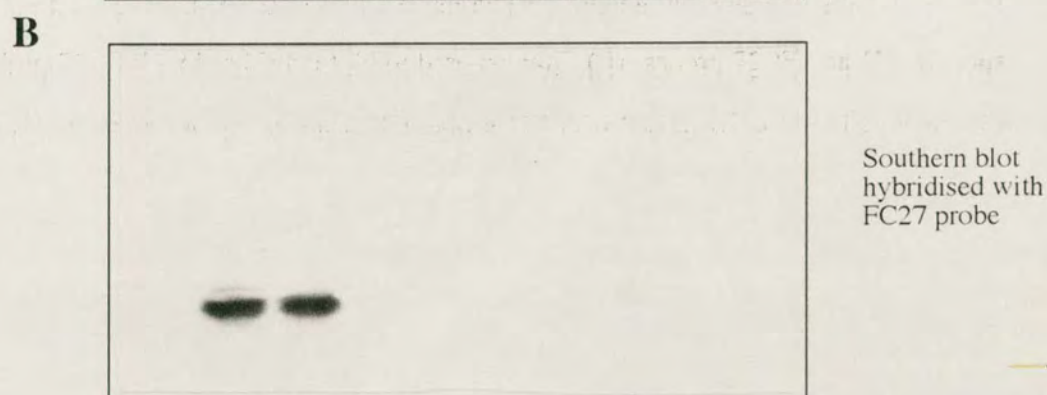
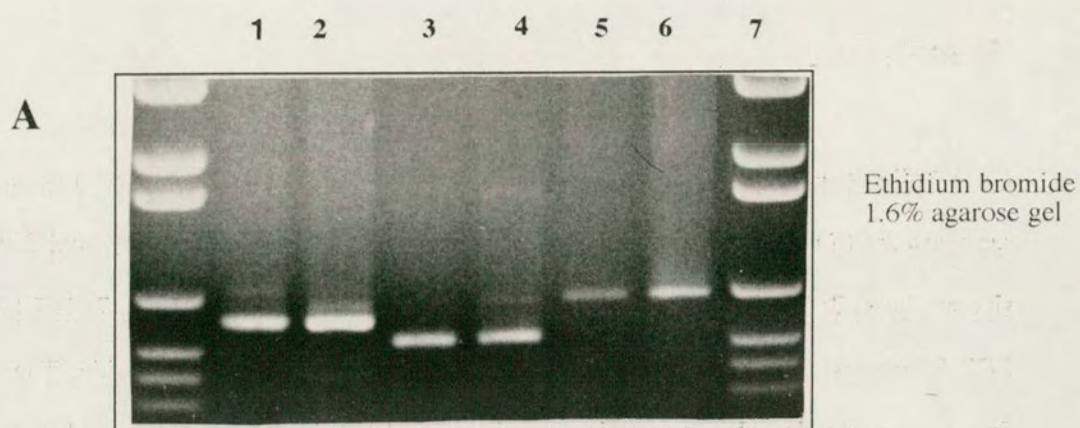
In one patient (123/89), two alleles of MSP-1 were seen in both primary and recrudescence samples, showing that this infection contained a mixture of at least two genetically distinct chloroquine-resistant parasite clones. In the other five patients, only single alleles of each gene were detectable, suggesting clonal-type infections in each case; however, it cannot be entirely excluded that these patients contained mixtures of parasites which by chance were identical at the MSP-1 and MSP-2 loci, but differed at other loci not examined in this work.



Table 19. Alleles of MSP-1 and MSP-2 genes of primary and recrudescence (R) *P. falciparum* in patients treated with chloroquine. Size: estimated size in base-pairs of PCR-amplified DNA fragments of each allele on agarose gels. Sequence: the probe which hybridizes with each PCR-amplified fragment. n.d. : not done.

Patient	Age (yrs)	Dates sampled	MSP-1 alleles	MSP-2 alleles
			Size/Sequence	Size/Sequence
107/89	30	25.10.89	470/Ro33	650/IC1
107/89-R		24.11.89	470/Ro33	650/IC1
123/89	17	31.10.89	540/MAD20+470/Ro33	540/IC1
123/89-R		23.11.89	540/MAD20 ⁺ 470/Ro33	540/IC1
101/90	8	7.10.90	530/K1	580/FC27
101/90-R		26.10.90	530/K1	580/FC27
103/90	15	20.10.90	470/Ro33	540/FC27
103/90-R		9.11.90	470/Ro33	540/FC27
120/90	50	29.10.90	470/K1	n.d.
120/90-R		13.11.90	470/K1	580/FC27
124/90	20	1.11.90	470/Ro33	n.d.
124/90-R		10.11.90	470/Ro33	540/IC1

Figure 19.

MSP-2 alleles in isolates from three patients classified by size and sequence. Primary and recrudescent (R) isolates from each patient were run side by side. Tracks: 1 and 8, DNA size markers. 2: 101/90. 3: 101/90-R. 4: 123/89. 5: 123/89-R. 6: 107/89. 7: 107/89-R. (A) PCR fragments separated on 1.6% agarose gel stained with ethidium bromide. The fragments were blotted on to a nylon membrane and hybridized separately with the allele specific IC1 and FC27 probes. (B) Film exposed to blot hybridised with FC27 probe. (C) Film exposed to blot hybridised with IC1 probe. (D) Schematic representation of the results.



Key:  IC1  FC27

5.5. Discussion

The high frequency of chloroquine resistant *P. falciparum* in Asar is not surprising since chloroquine resistance was reported in this region in 1987 (Bayoumi *et al.*, 1989). Pyrimethamine resistance had not been previously reported in this region: however, it is now clearly established. The presence of all possible combinations of *in vitro* responses to chloroquine and pyrimethamine and the high level of sensitivity to mefloquine suggests that resistance to each drug arises independently, and this is most probably caused by mutations or selection of unlinked genes. Therefore, it seems unlikely that multi-drug resistance to these three drugs is due to a single mutation in a gene such as the P-glycoprotein as suggested by Endicott & Ling, (1989) for multidrug-resistant (mdr) tumour cells. None of the chloroquine resistant clones tested had *Pfmdr1* gene amplification (Bayoumi *et al.*, 1993).

The use of pure clones of some isolates enabled a clear differentiation of parasite responses to these drugs in the village. A multiclonal isolate can have a phenotypic response different from its individual constituent clones. For example, isolate 106/89 had a high level of resistance to both chloroquine and pyrimethamine, but its clones had a lower level to both drugs; this was probably due to isolation of these clones from a muticlonal isolate which included other clones with a higher level. Alternatively, the highly resistant clones could be lost during the long period of *in vitro* cultures before the cloning process. The emergence of clone SUD 123/5 with a higher response to mefloquine than its original isolate could probably be explained by its having some selective advantage in culture.

Fluctuations in the frequencies of alleles of genes determining antigens within the village has been observed from year to year (chapter 4). This was possibly due to random genetic drift associated with a severe reduction in the parasite population size during the dry season. The observed stable frequency of chloroquine resistance suggests that the resistance to this drug is probably controlled by several genes, which additively exert different levels of response. It is clear that clones with a wide range of response ($0.2 \times 10^{-6} \text{M}$ to 1.610^{-6}M), co-exist together. An

alternative hypothesis is that the resistance could be due to a single gene as suggested by Wellemans *et al.* (1990), with two alleles at equal frequencies in the village. However, this hypothesis could not explain the existence of different clones with intermediate levels of response.

The changes in response to pyrimethamine from 1989 to 1990, may be explained by the likelihood that the phenotypic response to this drug is conferred by a single gene which varies in frequency from one season to another due to random drift and/or selection. *In vitro* there are clearly two levels of response; sensitive with MIC ranges between 10^{-8} M and 10^{-7} M and resistant with levels with MIC between 10^{-5} M and 10^{-4} M. However, occasionally there are some intermediate levels. Pyrimethamine acts selectively by inhibiting the enzyme dihydrofolate reductase (DHFR). Sequence data from pyrimethamine resistant and sensitive clones have indicated a role for amino acid 108 in the mechanism of resistance (Peterson *et al.*, 1988a). These authors have found that in the resistant parasites asparagine was present at this position, while in sensitive parasites either serine or threonine is present. These polymorphisms can be identified using mutation-specific PCR technique (Zolg *et al.*, 1990). It is unclear whether other mutations in DHFR confer different levels of resistance, or whether other genes may be involved in some isolates (Peterson *et al.*, 1990).

With regard to treatment of patients with chloroquine-resistant parasites, RII and RIII types of resistance are easy to diagnose and alternative antimalarial drugs can be prescribed immediately. However, symptoms caused by recrudescence R1 parasitaemias may frequently be confused by clinicians for other conditions, for which quite different treatments may be prescribed. Thus, patients infected with R1 chloroquine resistant *P. falciparum* may harbour their parasites for a long time, and these have an obvious epidemiological significance, especially if they are accompanied by gametocytaemia. Correct diagnosis of recrudescence R1-type parasites is therefore important, and has a practical impact on management of *P. falciparum* malaria in areas where resistance occurs.

In this study parasites which re-appeared in the blood of patients suspected of having chloroquine-resistant (R1) *P. falciparum* possessed the same alleles of MSP-1 and MSP-2 as the parasites of the corresponding primary infections. *In vitro* tests have confirmed that the primary parasites were slightly or moderately chloroquine-resistant when compared to a control drug-sensitive clone. Surveys of the parasites of the village during 1989 and 1990 showed the existence of 11 alleles of MSP-1 and 16 of MSP-2. This means that theoretically 176 (11x16) possible combinations of the alleles of each gene could occur among the parasites in the village. Although some alleles of each gene are more frequent than others, the probability that two parasite clones taken at random from the inhabitants will have identical alleles at these loci is very low. Among the 70 isolates examined during 1989 and 1990, only two had the same allele combinations, and these were from two sisters sleeping in the same house who had most probably been infected by the same mosquito on the same day. The rate and duration of transmission is low in the village; it is estimated that each villager may receive less than one infective bite per person per season (chapter 8). Thus, the probability that a single person will become re-infected from mosquitoes with parasites which have MSP-1 and MSP-2 alleles identical to those of the primary infection is also exceedingly low. For these reasons, it is concluded that the parasites re-appearing in the blood of the six chloroquine-treated patients were a genuine recrudescence of the primary parasites, and not a re-infection. This contrasts with the finding of recrudescence of mefloquine resistant parasites in patients from Thailand, where the recrudescence parasites were found to be different from the primary parasites (Pinswasdi *et al.*, 1987). This could be due to the fact that transmission intensity is higher in Thailand and people with multiclonal infections often have greater number of clones, which could be a mixture of sensitive and resistant parasites to mefloquine (Thaithong *et al.*, 1984).

Chapter 6. Genetic structure of *Plasmodium falciparum* population in a village in Tanzania.

6.1 Summary

The genetic structure of a population of *Plasmodium falciparum* has been examined in a village in Tanzania. Blood stage parasites and single oocysts in wild-caught mosquitoes were examined by PCR and allele-specific probes. 17 alleles of the merozoite surface protein MSP-1 and 23 of MSP-2 were detected by the polymerase chain reaction (PCR) among the blood parasites of the inhabitants. Most infections contained mixtures of genetically distinct parasite clones. PCR was then used to examine individual *P. falciparum* oocysts, the products of fertilisation events, in wild-caught mosquitoes. 45 out of 71 oocysts were heterozygous for one or both genes, showing that cross-mating between clones was taking place frequently in the mosquitoes. The frequency of the oocyst genotypes showed that random mating events occurred within mosquitoes between gametes belonging to different parasite clones.

6.2. Materials and Methods used.

6.2.1. Samples and Methods

56 *P. falciparum* isolates (each of 50-200 µl of blood) were collected in heparinised microvette tubes, from individuals in the village, aged between 2 and 41 years. All the samples were collected on three days, 21st, 24th & 25th June 1991. The isolates were processed and stored deep-frozen (section 2.2.2.). Later, DNA was isolated as described (section 2.6.2.).

Mosquitoes, *An. gambiae* and *An. funestus*, were collected using the indoor resting method from huts in the village, between June 10th and 23rd, 1991. They were kept in an insectary for 5-7

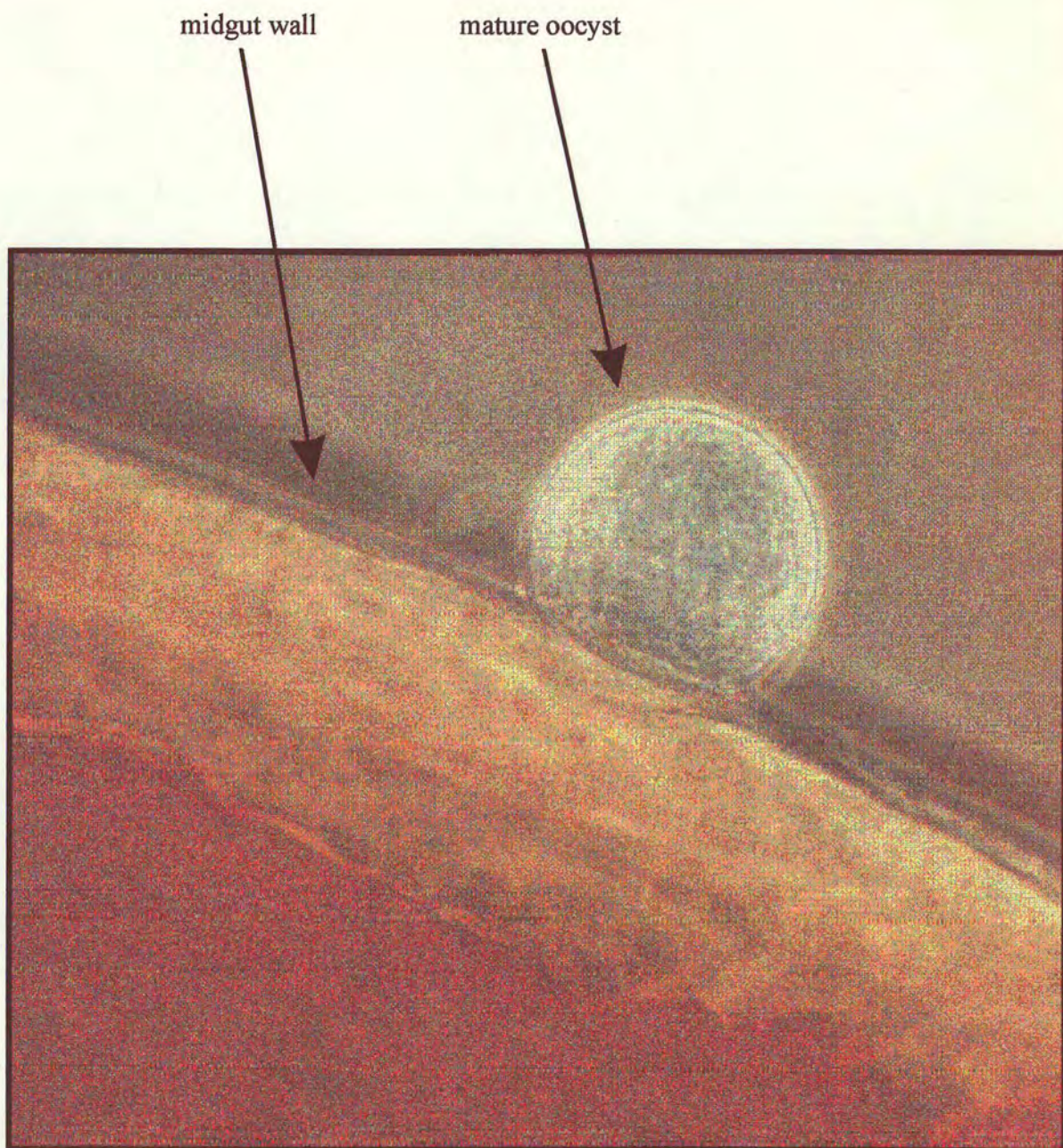


Figure: 20

A mature oocyst on mid-gut surface of a mosquito.

Photograph taken by: Dr Patricia Graves

days, then dissected and their midguts examined for oocysts. Midguts infected with single oocysts (Figure 20) were immediately transferred into 50 µl of lysis buffer supplemented with proteinase K and then incubated at 55°C for one hour. Oocysts were stored at -20°C until they were transported to Edinburgh. Later, DNA was isolated as described before (section 2.2.3.).

Alleles of MSP-1 and MSP-2 in blood and oocysts were determined using PCR and allele-specific oligonucleotide probes (section 2.6.).

6.2.2. Population genetic analysis

6.2.2.a Hardy-Weinberg analysis

The genetic structure of the parasite population was first examined by testing for deviation from Hardy-Weinberg equilibrium by alleles in oocysts. Out of 76 oocyst studied, 60 which were successfully typed for both MSP-1 and MSP-2 are included in this analysis. The alleles were classified only by their DNA sequences, i.e. K1-, MAD20- and Ro33-types for MSP-1, and IC1- and FC27-type for MSP-2, ignoring size differences. This was done because the total oocyst sample size (60) was small when considering the numbers of alleles based on size as well as sequence, which would have given too large a number to be treated by this type of analysis.

Are
remains
different?

The observed allele frequencies of each gene were first calculated from the 60 oocyst genotypes. The genotypes for each locus were determined and then compared with Hardy-Weinberg expectations.

6.2.2.b Analysis allowing substructuring of parasites in people

This part of the work was carried out in collaboration with Dorothy Currie, ICAPB, Edinburgh. A mathematical model was formulated to analyse the genetic structure of this *P. falciparum*

population. Taking into account the parasite load and the number of clones in different people in the village (Table 21). The parasites are not randomly distributed between people, but "substructured" with varying numbers of clones in each human host. The distribution has been modelled as being negative binomial in form. This is a discrete distribution with variance always greater than the mean. The shape of the distribution is determined by two parameters, the mean number of clones per patient, and the variance in the distribution of clones in infected people. The distribution can be summarised by the proportion of people with single clones, and the mean number of clones per person.

In the model, allele frequencies were allowed to vary from 0 to 1 for each locus. Frequencies of the possible oocyst genotypes were generated using a range of allele frequencies given the number of clones present in people (from the negative binomial distribution), by multinomial sampling theorem. The oocyst genotype frequencies generated using these parameters were then compared with those actually observed by maximum likelihood. The support range of each parameter is given, i.e. 95% confidence interval (see Appendix 1 for model details).

6.3. Results

6.3.1. Allelic polymorphism of MSP-1 & the MSP-2 in blood forms.

Fifty three *P. falciparum* isolates collected from inhabitants in the village were typed for both MSP-1 and MSP-2. 41 isolates gave positive PCR products for both genes and 12 isolates gave a positive reaction for one or other. Alleles of each gene were determined by both size of their PCR-amplified fragments and by their sequences using allele-specific probes. The actual number of alleles of each gene detected by this means is almost certainly an underestimate, since further minor differences in the number of repeats and sequences most probably exist which could be revealed only by sequencing of the PCR-amplified fragments.

17 alleles of MSP-1 (Fig. 23a) and 23 of MSP-2 (Fig. 25a) were found among the blood samples examined, distinguishable by both size and/or sequence. Using our primers the size of the MSP-1 fragments ranged from 410 to 600 bp. The three sequence variants of block 2 of MSP-1 were present among the alleles, 44% of alleles in infected individuals being K1-type, 26% MAD20-type and 30% RO33-type. For MSP-2, the amplified fragments ranged between 410 and 680 bp. 50% of the isolates had sequences of IC1-type and 50% of FC27-type.

Numerous combinations of alleles of the two genes were found among these isolates. Classifying the alleles by sequence type only, 11 of the possible 18 combinations were detectable. In at least 11 isolates which had multiclonal infections, it is possible that some of the other 7 combinations were also present (Table 20).

Table 20. Observed combinations of alleles of the MSP-1 & the MSP-2 gene based on sequence among 41 isolates of Michenga. which gave positive PCR product for both genes,

Number of isolates	MSP-1			MSP-2	
	K1	MAD20	RO33	IC1	FC27
3	+	-	-	+	-
0	+	-	-	-	+
7	+	-	-	+	+
1	+	+	-	+	-
0	+	+	-	-	+
2	+	+	-	+	+
2	+	-	+	+	-
1	+	-	+	-	+
5	+	-	+	+	+
1	-	+	-	+	-
0	-	+	-	-	+
2	-	+	-	+	+
0	-	+	+	+	-
2	-	+	+	-	+
0	-	+	+	+	+
0	-	-	+	+	-
0	-	-	+	-	+
2	-	-	+	+	+
3	+	+	+	-	+
11	+	+	+	+	+

+ presence of allele, - absence of allele

6.3.2 Multiclonal infections

45 out of 53 blood samples (85%) were found to harbour multiclonal infections, identified by the presence of two or more alleles of one or both genes (Table 20 and 21; Fig. 21). Since the blood forms are haploid, this is an indication of the presence of more than one parasite genotype. The three sequence variants of MSP-1 and the two of MSP-2 co-existed together in 11 individuals, indicating the presence of a minimum of 3 clones and a maximum of 6 clones in each of these people (Table 21., Figure 21). Taking all the isolates together, the mean number of clones per person is calculated to be between 2.2 and 3.2. At least 43% of the isolates contained a minimum of more than three clones each.

Table 21. The minimum and maximum number of clones per isolate within 53 isolates collected from the village. Maximum detectable=6.

Number of clones per isolates			
No. of isolates	Minimum	Maximum	Mean
11 (21%)	3	6	4.5
9 (17%)	2	4	3
3 (6%)	3	3	3
22 (41%)	2	2	2
8 (15%)	1	1	1
mean	2.2	3.2	2.7

Figure 21.

Alleles of MSP-2 gene in *P. falciparum* isolates from different people in Michenga detected by PCR amplification. Molecular size markers on the outer tracks are indicated in base pairs (bp). Note presence of two fragments in tracks, 2,4,6 and three in tracks 3 and 5, showing infections with parasites with different alleles

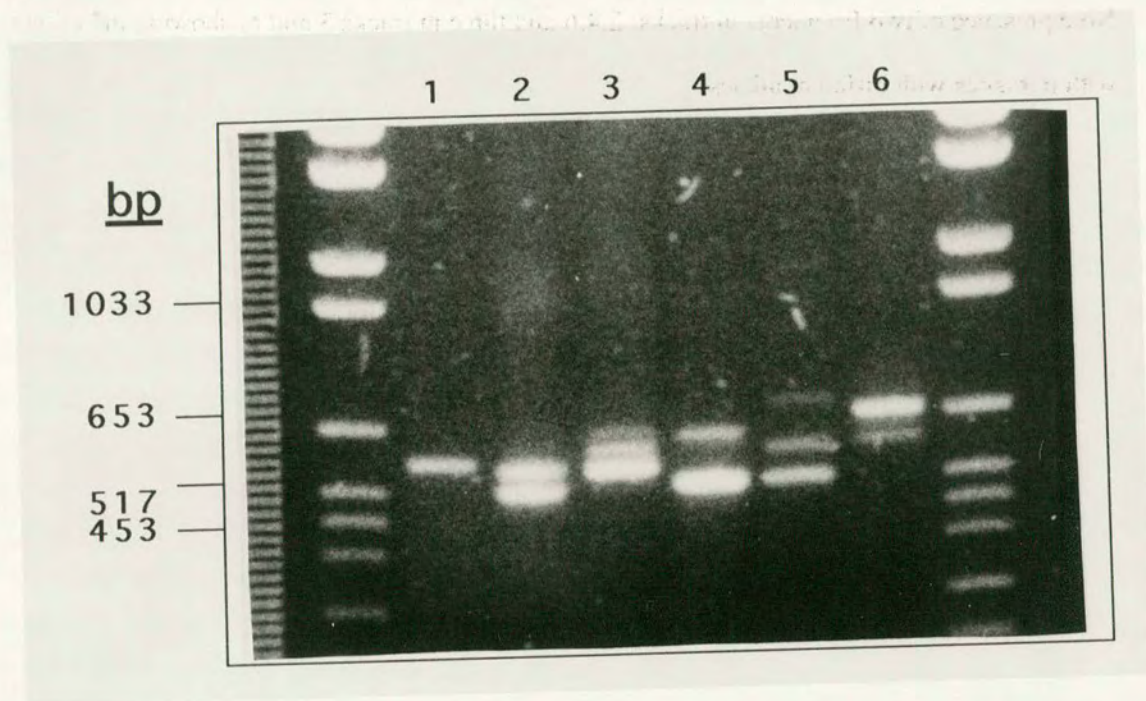
1. The first step in the process of DNA sequencing is the preparation of a DNA template. This is typically done by isolating the DNA from the organism of interest and then amplifying it using PCR. The amplified DNA is then purified and quantified.

2. The second step is the setup of the sequencing reaction. This involves the addition of the DNA template, sequencing primers, and sequencing reagents to a reaction mixture. The reaction is then incubated at a specific temperature for a set period of time.

3. The third step is the electrophoresis of the sequencing reaction products. This is done using a denaturing polyacrylamide gel (PAGE). The DNA fragments are separated based on their size, with smaller fragments moving further than larger ones.

4. The fourth step is the visualization of the DNA bands. This is typically done using a fluorescent dye that binds to the DNA. The bands are then imaged using a fluorescence reader or a gel imager.

5. The final step is the analysis of the sequencing data. This involves comparing the DNA sequence obtained from the sequencing reaction to a reference sequence. This can be done using software tools or by manual inspection of the gel image.

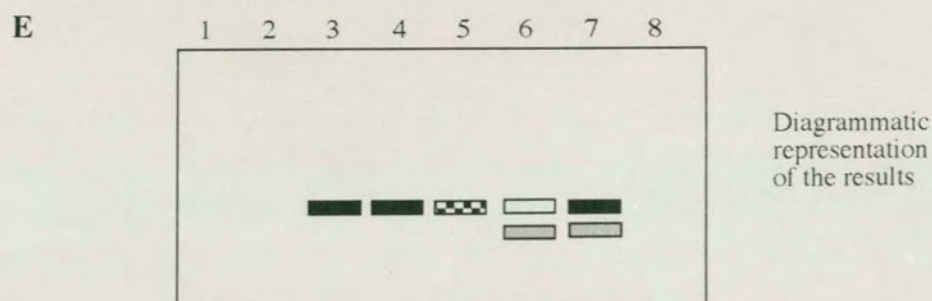
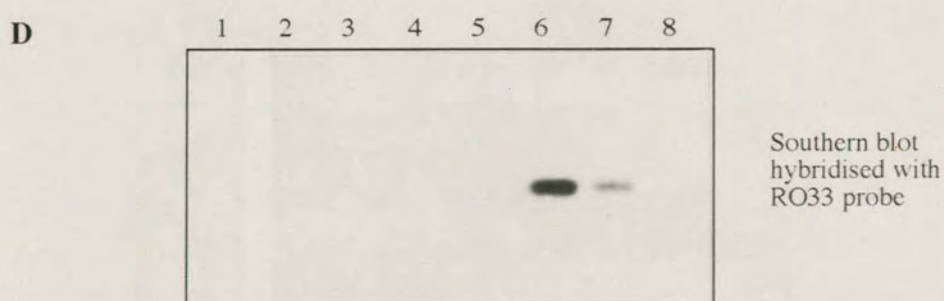
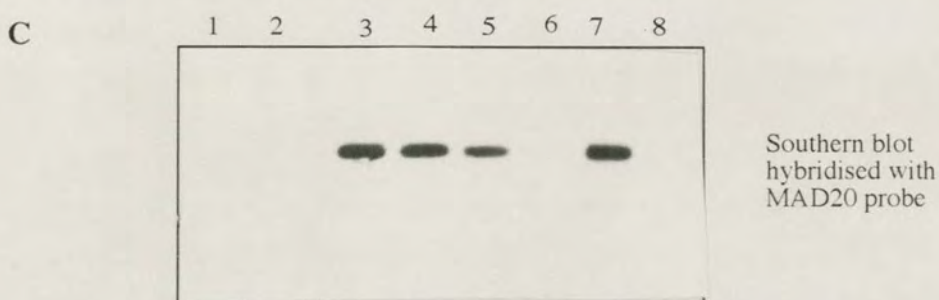
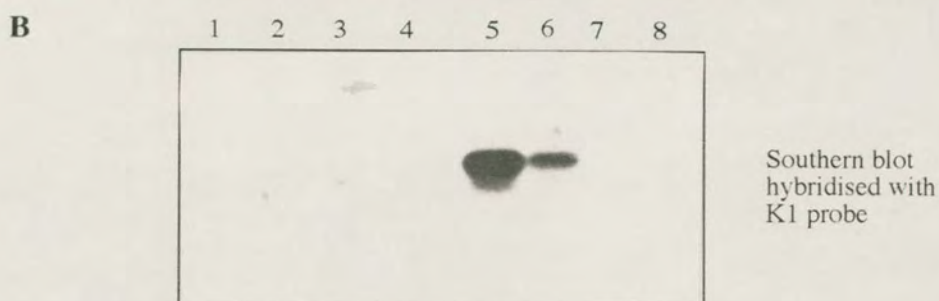
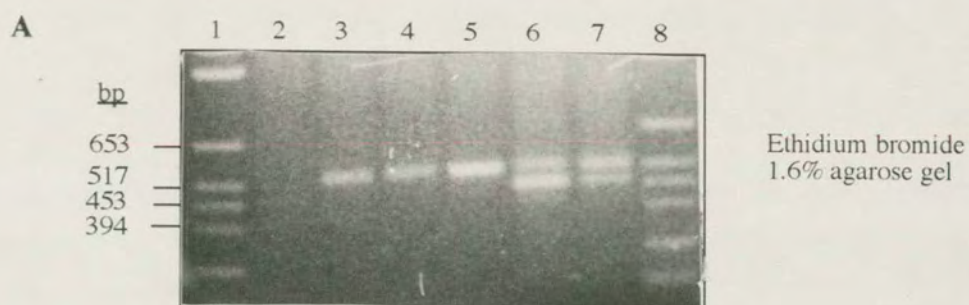


6.3.3 Genotype diversity of oocysts in wild mosquitoes.

168 out of 1136 mosquitoes (15%) collected from selected households in the village over three weeks developed oocysts after being kept for 5 to 8 days in the insectary. 79 (47%) of these were infected with single oocysts; the rest each had more than one oocyst. 76 midguts infected with single oocysts were processed for typing of MSP-1 and MSP-2. 71 of these produced PCR-amplified band for one or both genes, 60 of which were typed successfully for both genes. The oocyst genotypes were found to be highly diverse. 16 alleles of MSP-1 (Figures. 22 & 23.b) and 24 of MSP-2 (Figures. 24 & 25.b) were identified. All the alleles of MSP-1 found in the blood were seen in the oocysts, except for one rare allele (580/MAD20) seen in one blood sample but not in the oocysts (Figure 22). All MSP-2 alleles seen in the blood samples were detected in the oocysts, except one (IC1/410) found in two oocysts but not in the blood samples (Figure 25). The sequence variants, K1, MAD20 & RO33 of MSP-1 and the IC1 and FC27 of MSP-2 were all present in the oocysts. All the 18 possible two-locus genotypes based on these sequences were identified except three which involve rare alleles (Table.22).

Figure 22.

Alleles of MSP-1 gene classified by PCR and sequence specific probes, in oocysts from Michenga. PCR-amplified fragments separated by electrophoresis (panel A). Southern blots of these fragments were hybridized with allele-specific probes K1, MAD20 and RO33 (panel B, C and D respectively). Tracks 1 and 8 are size markers, tracks 2-7 are oocyst samples. Note oocysts in tracks, 5, 6 and 7 are heterozygous, having two alleles. The two alleles of the heterozygous oocyst each differs by sequence only (track 5), or both size and sequence (track 6 and 7). Panel E shows a diagrammatic representation of the results.

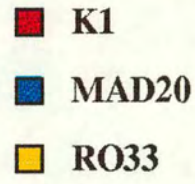


Key:

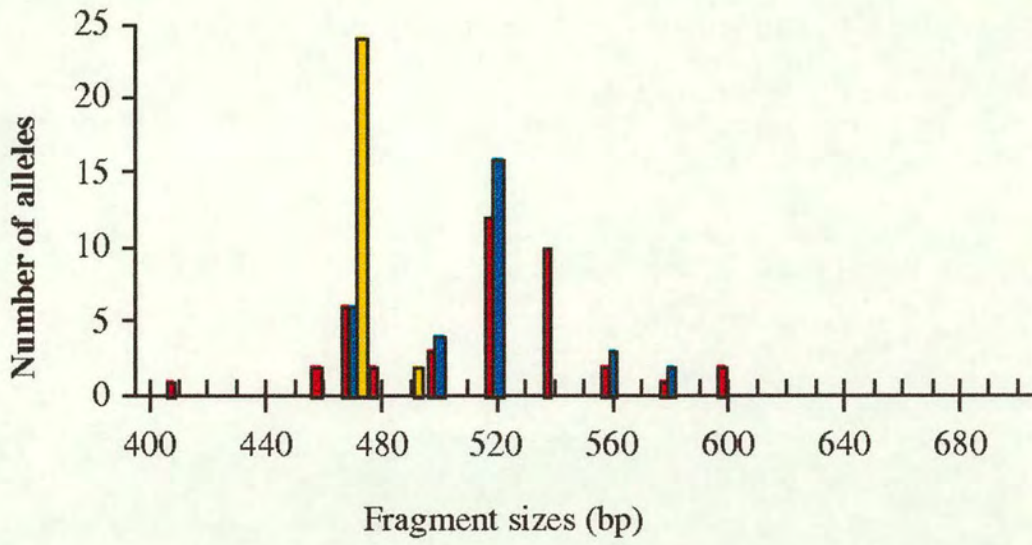
K1
 MAD20
 RO33
 K1 and MAD20

Figure 23.

Alleles of the MSP-1 gene in Michenga village, classified by size of their PCR-amplified fragments (bp) and sequence of these fragments (K1, MAD20, RO33) . (A) alleles of the blood forms among 53 isolates (B) alleles in 71 oocysts; the numbers are based on two alleles of each gene per oocyst, assuming that each oocyst is derived from a single diploid zygote.



(A) Blood



(B) Oocysts

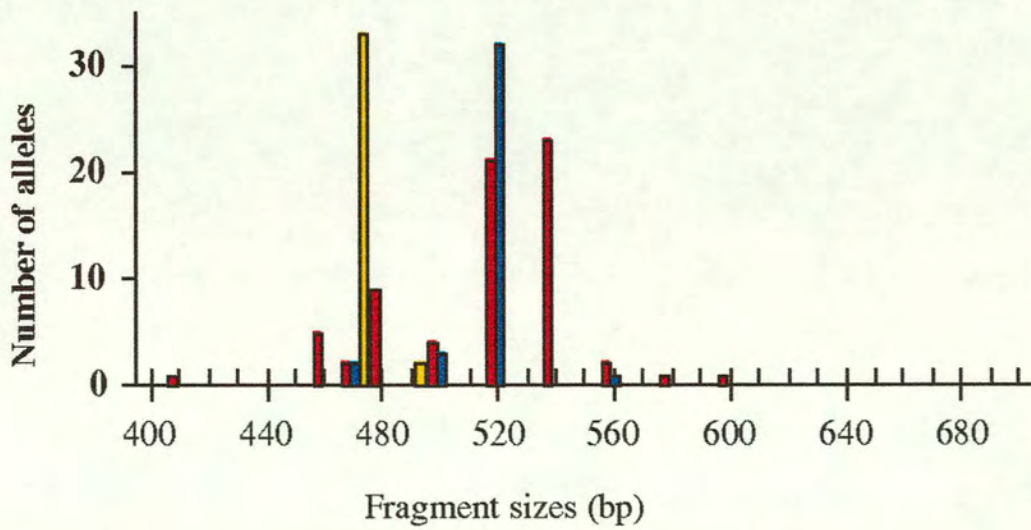


Table 22. Genotypes of 60 oocysts in mosquitoes in Michenga. The observed numbers of two-locus genotypes are shown.

Observed Number of oocysts	<u>Expected genotype</u>				
	K1 (0.53)	Mad20 (0.22)	RO33 (0.25)	IC1 (0.567)	FC27 (0.433)
8	+	-	-	+	-
5	+	-	-	-	+
4	+	-	-	+	+
3	+	+	-	+	-
4	+	+	-	-	+
5	+	+	-	+	+
5	+	-	+	+	-
5	+	-	+	-	+
7	+	-	+	+	+
3	-	+	-	+	-
3	-	+	-	-	+
0	-	+	-	+	+
2	-	+	+	+	-
0	-	+	+	-	+
1	-	+	+	+	+
4	-	-	+	+	-
0	-	-	+	-	+
1	-	-	+	+	+

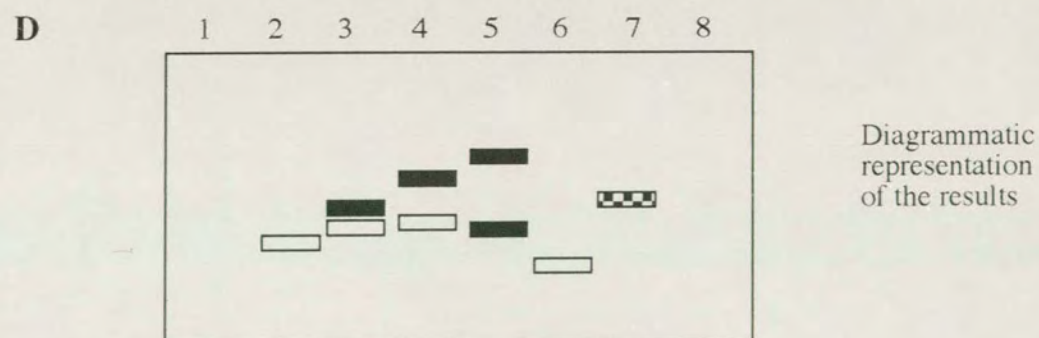
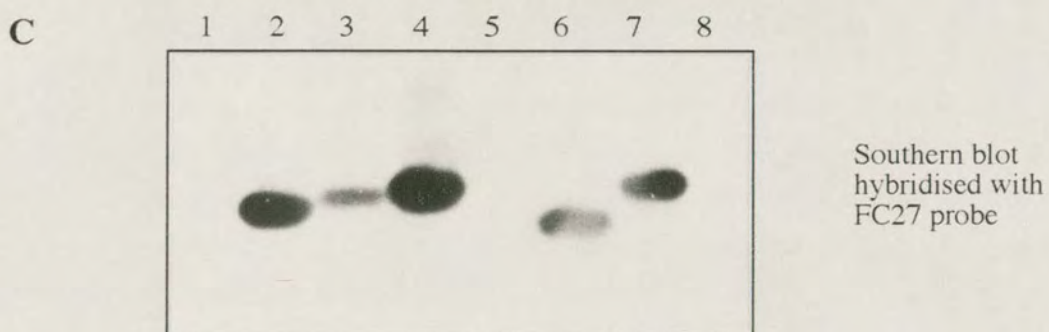
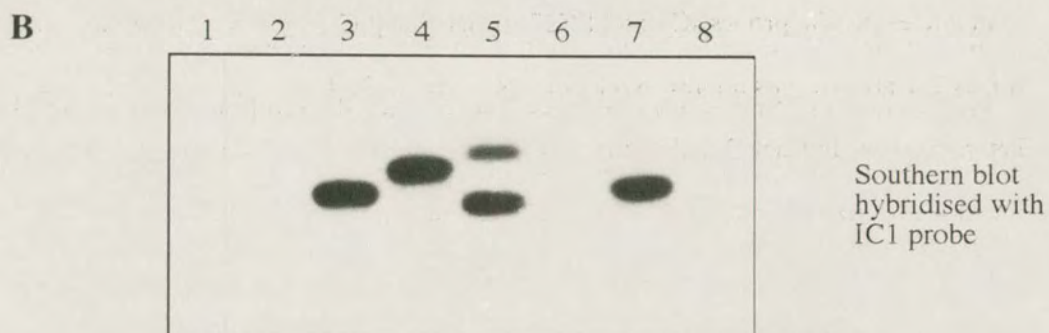
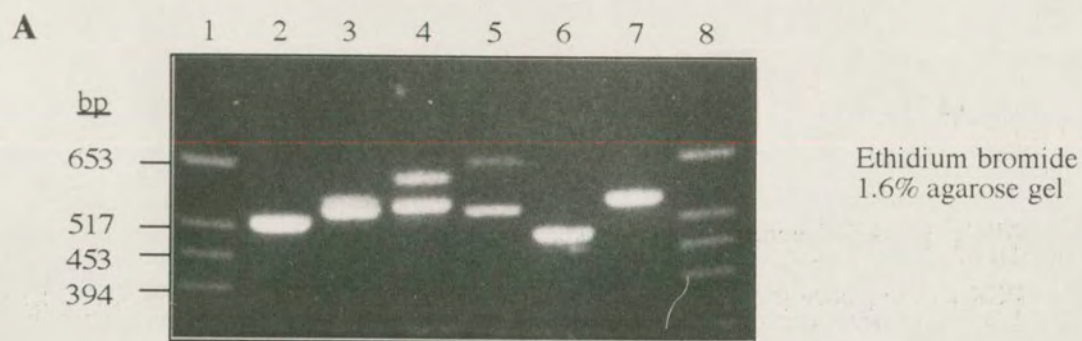
Observed frequencies of each allele in brackets

6.3.4. Frequency of hybrid oocysts.

35 of 71 individual oocysts examined for the MSP-1 were found to contain two unlike alleles of this gene, identified by the size and/or the sequence of the amplified region (Table 23; Figure 22). Similarly, 31 out of 60 oocysts examined for MSP-2 contained two unlike alleles of this gene (Table 23; Figure 24). Taking both genes into account, 45 out of 71 (63%) of the oocysts were found to be heterozygous for one or both genes.

Figure 24.

Alleles of MSP-2 gene of *P.falciparum*, classified by PCR and sequence specific probes, in 6 oocysts from Michenga. PCR-amplified fragments separated by electrophoresis (panel A). Southern blots of these fragments were hybridized with allele-specific probes IC1 and FC27 (panel B and C). Tracks 1 and 8 are size markers, tracks 2-7 are oocyst samples. Note oocysts in tracks 3, 4 and 5 are derived from heterozygotes. In track 3 and 4, the two alleles each differ by both size and sequence (track 3 and 4), by size only (track 5) or by sequence only (track 7). The oocyst in track 2 has derived from homozygotes with two identical alleles. Panel D shows a diagrammatic representation of the results.



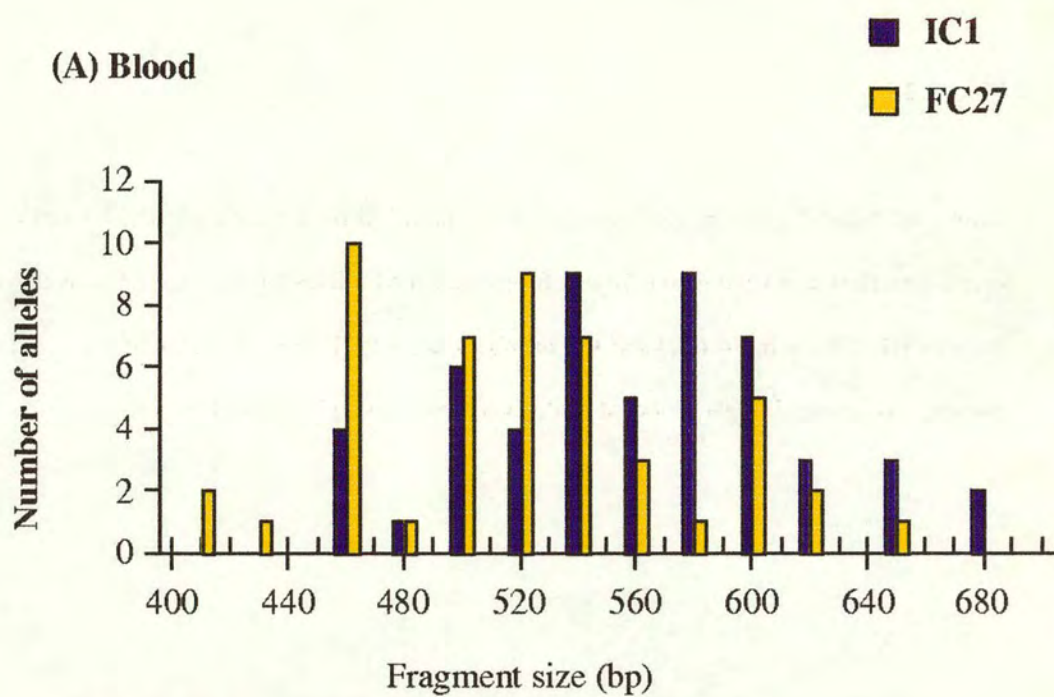
Key:

■ IC1 □ FC27 ▨ IC1 and FC27

Figure 25.

Alleles of MSP-2 gene in Michenga village, classified by size of their PCR-amplified fragments (bp) and sequence of these fragments. (A) alleles of the blood forms among 53 isolates (B) alleles in 64 oocysts; the numbers are based on two alleles of each gene per oocyst, assuming that each oocyst is derived from a single diploid zygote.

(A) Blood



(B) Oocysts

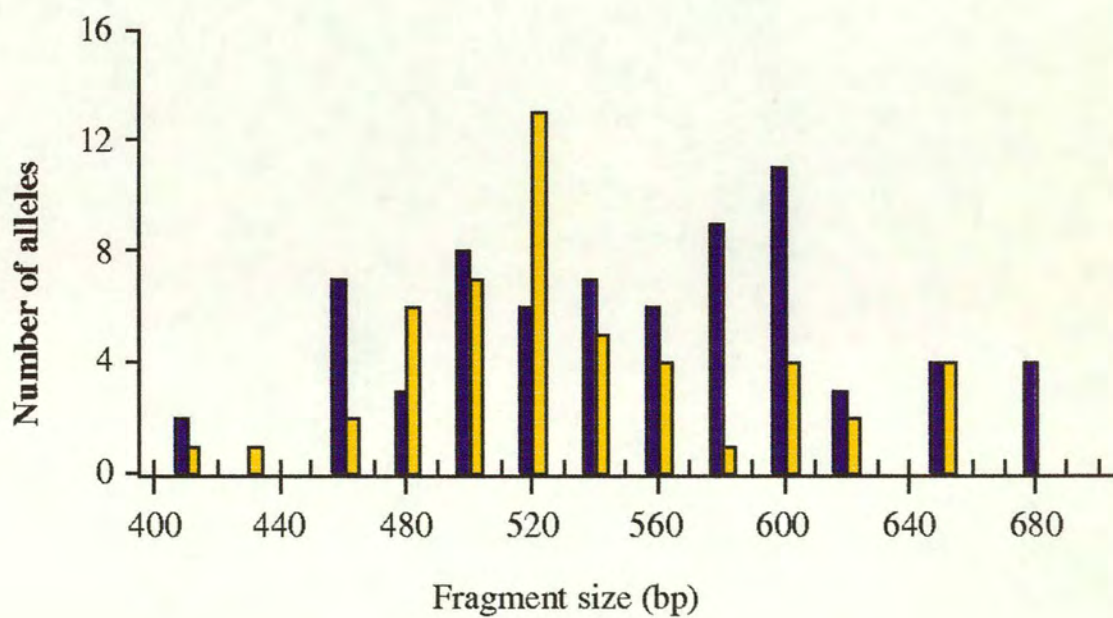


Table 23. Analysis of 76 oocysts collected from wild-caught mosquitoes of Michenga.

oocysts number		Heterozygous	Homozygous
MSP-1 gene	71	35 (49%)	36 (51%)
MSP-2 gene	60	31 (52%)	29 (48%)
MSP-1 & MSP-2	71	45 (63%)	26 (37%)

6.3.5 Population genetic analysis

(i) Hardy-Weinberg analysis

Allele frequencies of both genes were first calculated among the 60 oocysts that were typed successfully for both genes. The two-locus genotypes of the oocysts were determined and their frequencies were compared with those expected for genes in Hardy-Weinberg equilibrium. Results show that fewer heterozygotes were seen than would have been expected for a random mating population (Table 24). There was a deficit of heterozygous forms for both genes. For MSP1, 32 heterozygotes were found, when the expected number for a randomly mating population would be 36.68. For MSP-2, the deficit was more marked, there being only 18 heterozygotes instead of an expected number of 29.47. The two-locus homozygotes and heterozygotes proportions were examined using the frequency of homozygosity at each locus, no significant difference was found between the two loci ($0.1 > P > 0.05$) (Table 25).

How does
this
fit with
the
Table 23

↓
difference is
that 23 include
size as well as
sequence
polymorphisms.

(ii) Analysis allowing substructuring of parasites in people

This statistical model allows analysis of mating patterns within parasites "substructured" among the infected individuals in the village. Maximum likelihood estimates (and support) generated from the model summarise the shape of the distribution by the following:- (i) mean number of clones per person, 3.2 (2.6-4.0), (ii) the proportion of people with single clones, 0.07 (0.00-0.23) and (iii) estimates of the allele frequencies are K1, 0.6 (0.5-0.70), MAD20, 0.20 (0.15-0.25) and IC1, 0.6 (0.45-0.7), (maximum Ln likelihood= -160.95). The model predicting an average number of 3.2 clones per person, and a proportion of clonal infections of up to 23%, accords well with the findings of the PCR analysis of the blood samples collected from the village population, with a range of 2.2 to 3.2 single genotypes per person and 15% clonal infections (Table 21).

There is no significant difference between the observed numbers of homozygous and heterozygous oocysts at both MSP1 and MSP2 loci, and the numbers predicted from the maximum likelihood estimates ($G_1=3.8$, $df=1$, $0.1 > P > 0.05$) (Table 26).

Table 24. Comparison of observed and expected number of oocysts genotypes assuming Hardy Weinberg predictions.

(A) MSP-1 :-

K1/K1 (p ²)	MAD20/MAD20 (q ²)	Ro33/Ro33 (r ²)	K1/MAD20 (2pq)	K1/Ro33 (2pr)	MAD20/Ro33 (2qr)	Total
(16.54)	(3.04)	(3.75)	(14.18)	(15.75)	(6.75)	60
17	6	5	12	17	3	60

In brackets expected numbers, G=5.69, df=3, 0.2>P>0.1

(B) MSP-2 :-

	IC1/IC1 (x ²)	FC27/FC27 (y ²)	IC1/FC27 (2xy)	Total
Exp.	19.27	11.27	29.47	60
Obs.	25	17	18	60

G=9.24, df=1, 0.005>P>0.001

(C) MSP-1 and MSP-2.

MSP-1				
		Homozygous Observed (Predicted) (p ² +q ² +r ²)	Heterozygous Observed (Predicted) (2pq+2pr+2qr)	Total
MSP-2:	Homozygous (x ² +y ²)	23 (11.87)	19 (18.66)	42
	Heterozygous (2xy)	5 (11.46)	13 (18.06)	18
Totals		28	32	60

(G=14.27, df=1, P<0.005), q(0.525), p(0.225), r(0.250), x(0.567) and r(0.433) are the observed gene frequencies of K1, MAD20, RO33, IC1 and FC27 respectively.

Table 25. Observed homozygosity at each locus used to calculate expected two-locus homozygosity.

		MSP-1	
		Homozygous	Heterozygous
		Observed (Predicted)	Observed (Predicted)
MSP-2:	Homozygous	23 (19.6)	19 (22.4)
	Heterozygous	5 (8.4)	13 (9.6)
Totals		28	32
			60

G1= 3.79, df=1, 0.1>P>0.05. MSP-1 homozygotes proportions (28/60), MSP-2 homozygotes proportions (42/60). MSP-1 heterozygotes proportions (32/60), MSP-2 heterozygotes proportions (18/60).

Table 26. Analysis of oocyst data assuming random mating within substructured population. The maximum likelihood parameter values were used to generate expected proportions of homozygotes and heterozygotes at the two loci to compare with the expected number in each class.

		MSP-1	
		Homozygous	Heterozygous
		Observed Predicted	Observed Predicted
MSP-2	Homozygous	19 17.94	19 14.92
	Heterozygous	5 9.29	13 14.05

G-test indicated no significant difference between the observed and predicted numbers, (G3=3.8, 0.3>P>0.5).

6.4. Discussion.

Tibayrenc *et al.* (1990; 1991) have proposed that there is non-random association between genes in natural populations of *P. falciparum*, which implies that cross-mating and genetic recombination are rare. However, this study has shown clearly that crossing between parasites occurs frequently during the natural course of transmission. 63% of individual zygotes (oocysts) examined were found to be hybrids (heterozygous) resulting from cross-mating between genetically different

gametocytes. Those oocysts were identified by the presence of two alleles of either MSP-1 and/or MSP-2. The results of this study have provided, for the first time, firm evidence for sexual reproduction within a natural population of *P. falciparum*.

The alleles used in the population genetic analysis were those identified only by sequence. It would have been possible to group alleles, on the basis of size alone, into "bins" around an estimated size (Weir 1992), but such grouping would be somewhat arbitrary, due to technical difficulties in estimating the sizes of regions containing variable numbers of tandem repeats (VNTR). On the other hand, the allele-specific sequence used in this work allowed the grouping of alleles on the basis of their sequence. The sequences chosen as sites for allele-specific probes of the two genes are conserved among all alleles studied so far, and every allele has one or other of these sequences (Miller *et al.*, 1993; Smythe *et al.*, 1991). Neither of the two genes has been reported to have intragenic recombinant sites in the positions of the probes used here.

An examination of oocyst genotypes in mosquitoes which had deliberately fed on a mixture of two genetically distinct clones of *P. falciparum* allowed direct application of Hardy-Weinberg analysis. This indicated that random mating between these clones occurred (Ranford-Cartwright *et al.*, 1993). However, care must be taken when using Hardy-Weinberg predictions to analyse natural populations of *Plasmodium*. Departure from Hardy-Weinberg expectations is likely because the parasite population is partitioned or "substructured" among infected individuals. Although many genotypes exist in the whole village, each infected individual contains only a limited number of clones.

Individual contact between parasites depends entirely on the mosquito vector. In nature, non-random contact between the mosquito vector and the human host, and infrequent mixed blood meals by mosquitoes have been reported (Dye & Hasibeder, 1986; Boreham *et al.*, 1979). Thus, mating occurs almost exclusively between gametes from the same individual, and these will not be representative of the population as a whole. This will also tend to increase the number of

homozygotes observed in mosquitoes. Samples collected across a series of randomly mating sub-populations can thus give rise to an apparent deficit of heterozygotes (Wahlund effect).

The oocyst genotype frequencies found in this work show an excess of homozygotes if one assumes no population subdivision (table 24). The deficit of heterozygotes is significant for MSP-2, and for the two locus-genotypes, However, the MSP-1 did not show that level of deficit. That was probably due to chance, given the small sample sizes involved. However, when the two-locus homozygotes and heterozygotes proportions were examined using the frequency of homozygosity at each locus, no significant difference was found between the two loci (Table 25). An analysis using all alleles based on size as well as sequence using Monte Carlo simulation with random pairing of alleles has also shown a highly significant deficit of heterozygotes on both loci (W.G. Hill, personal communication). However, the diploid genotype frequencies observed can be adequately explained by random mating between gametes from a substructured parasite population. Such non-random mating caused by population subdivision, rather than preferential selfing, does not necessarily imply clonal reproduction. Single clonal infections give rise to identical gametes which undergo self-mating, producing only homozygous zygotes. When an individual harbours multiclonal infections, homozygous zygotes can still be produced between gametes of the same type, together with heterozygous zygotes produced by cross-mating between different clones. Only if all individuals are randomly infected with all genotypes would conventional Hardy-Weinberg proportions be expected.

The observed random mating within the parasite population of Michenga agrees with previous reports from many endemic countries which showed a high level of diversity within local parasites, suggesting that sexual reproduction must be frequent among them (reviewed by Walliker, 1991). Analysis of alleles of enzymes and antigens in parasite populations in the Gambia have suggested that these genes were in linkage equilibrium (Carter & Voller, 1975; Conway & McBride, 1991). Similarly parasite populations in Sudan, Thailand, Zimbabwe and Brazil were found to be consistent with frequent sexual recombination (Babiker *et al.*, 1991a; Maynard Smith *et al.*, 1993;

Creasey *et al.*, 1990). The two relevant consequences of sexual reproduction for any organism are segregation of alleles at a single locus and recombination between genes at different loci. In contrast to the views of Tibayrenc *et al.* (1990; 1991) it is clear that the two processes are frequent within the parasites of Michenga.

Chapter 7. Dynamics and polymorphism of *Plasmodium falciparum* at the household level in a village in Tanzania

7.1. Summary

This chapter investigates the extent of genotypic polymorphism in *P. falciparum* at the household level, and examines whether the population of parasites is homogeneously mixed or consists of partially isolated groups among widely dispersed households. *P. falciparum* blood forms from people and individual oocysts from mosquitoes were collected simultaneously from four households over a period of two weeks. No evidence of genotype clustering or significant variation in allele frequencies was observed between the studied households.

7.2. Material and Methods used.

The study was carried out in a small hamlet (Kininina) near Michenga village, Tanzania (section 2.1.2.). The hamlet contains four family households each consisting of two to four small huts grouped 7-10 meters apart. Each family was given a serial number 8001 to 8006, and each hut a sub-number. The members of each family varied between 5 and 11. Households with more than one room (hut) are treated here as a single household. For example house number 8004/1-4 is in fact four huts, where a husband, three wives and children live together. However, house 8001/1 is a single hut where a husband, wife and two children live. These houses are scattered within a rice-growing area approximately 1 km apart.

Fingerprick blood samples (50-700 µl) were collected from all inhabitants of the four households who had *P. falciparum* infections. This collection was done on one day, 21st June 1991. Samples were cryopreserved (section 2.3.1.). Later, DNA was isolated from blood samples as described in section 2.6.2.

Mosquitoes, *An. gambiae* and *An. funestus*, were collected using the indoor resting method (section 2.2.3.) on a daily basis from the four houses, between June 10th and 23rd, 1991. The

mosquitoes were kept in an insectary for 5-7 days before being dissected, and midguts containing single oocysts were processed (section 2.6.3.). Later, DNA was isolated from individual oocysts as described (section 2.6.3.). In addition, 12 blood fed female *Anopheles* mosquitoes were collected on two consecutive days on 24th and 25th, June 1991, from household 8001/1. The abdomens of each mosquito were cut separated from the rest of the body and individually processed as described in section 2.6.3.

PCR was used to amplify regions of MSP-1 and MSP-2 (section 2.6.4). The amplified fragments were separated by electrophoresis (section 2.6.4.2), blotted to nylon membranes and classified on the basis of their sequences using allele-specific probes (section 2.6.6.).

Statistical analysis

Due to the small numbers of oocysts collected in each household, statistical analysis was done only using the sequence-types of the PCR product of each gene. To investigate the possibility of spatial clustering of parasites within the Kininina hamlet, the observed frequencies of alleles of the two genes were compared to those of the village as a whole. Further more, the allele frequencies in each household were compared to those found in the whole village.

7.3. Results.

7.3.1. Genotypes of oocysts infecting mosquitoes in different households.

The overall oocyst rate within the village at the time of the study was approximately 15% of mosquitoes studied. However, there were variations between the houses studied, ranging from 8% to 17% (Table 27).

The 2-locus genotypes of the oocysts are given in Table 28. Extensive polymorphism was observed within each household, as shown in Table 28. For example, 8 alleles of MSP-1 and 10 of MSP-2 identified by size and sequence of each gene were detected in household 8001/1-2. Although high, this was less than the number of alleles found in the whole village (17 and 24 of MSP-1 and MSP-2 respectively)(chapter 6). No two oocysts with identical genotypes were found in any household. Even oocysts obtained from mosquitoes collected on the same night in the same household were found to be different. Heterozygous oocysts identified by the presence of two alleles of MSP-1 and/or MSP-2, and homozygous oocysts, with identical alleles, were also detected within each of the studied households. The homozygous oocysts found in each household were also of different genotypes.

Table 27. Oocyst rate in the studied households of Kininina hamlet.

House Number	Uninfected mosquitoes	Infected mosquitoes	Total
8001/1-2	323	60 (17%)	383
8003/1-2	96	11 (10%)	107
8004/1-4	347	31 (8%)	378
8006/1-4	73	10 (12%)	83

Table 28. 2-locus genotypes of oocysts collected from households in Kininina, Tanzania.

house number	Oocyst number	MSP-1 alleles			MSP-2 alleles		Collection Date
		K1	MAD20	RO33	IC1	FC27	
8001/1-2	If14	+(460)	-	-	+(600, 520)	-	11.6.91
	If15	-	+(500)	-	-	+(600)	"
	If25	-	+(520)	-	ND	ND	"
	If34	-	+(520)	-	ND	ND	12.6.91
	If42	+(520)	-	-	+(480)	+(620)	13.6.91
	If44	+(540)	-	-	+(620)	+(480)	12.6.9
	If50	-	+(500)	+(470)	+(560)	-	18.6.91
	If57	+(520)	-	-	+(580)	-	19.6.91
	If58	+(480)	+(480)	-	+(560)	+(520)	"
	If59	+(520)	-	-	-	+(480)	"
	If68	+(540)	+(520)	-	+(460)	-	20.6.91
8003/1-3	If9	-	-	+(470)	ND	ND	11.6.91
	If10	+(520)	-	+(470)	-	+(480)	"
	If11	+(540)	-	-	ND	ND	"
	If31	-	+(520)	-	+(680)	-	13.6.91
	If73	+(560)	+(520)	-	+(600)	-	20.6.91
8004/1-4	If22	+(540)	-	+(470)	ND	ND	12.6.91
	If23	+(540)	-	-	+(680)	-	"
	If32	+(480)	-	-	ND	ND	13.6.91
	If39	+(520)	+(520)	-	-	+(490)	12.6.91
	If40	+(500)	-	-	+(480)	+(620)	12.6.91
	If48	+(490)	-	-	+(580; 540)	-	17.6.91
	If66	+(520)	+(520)	-	+(540)	+(540)	20.6.91
	If70	+(540)	+(520)	-	-	+(540)	20.6.91
	If71	-	+(520)	+(470)	+(650; 540)	-	20.6.91
	If72	+(470)	-	+(470)	+(620; 560)	-	20.6.91
	If74	+(540)	-	+(470)	+(600)	+(450)	21.6.91
	If77	+(480)	-	-	-	+(490)	21.6.91
8006/1-4	If47	+(520)	-	+(470)	+(600; 540)	-	17.6.91
	If51	+(480)	-	+(470)	+(580; 540)	-	18.6.91
	If61	+(520)	-	+(470)	+(580)	+(520)	19.6.91
	If62	+(520)	-	-	ND	ND	19.6.91
	If63	+(540)	-	+(470)	+(620; 500)	-	19.6.91
	If64	+(520)	-	+(490)	ND	ND	19.6.91
	If65	+(520)	+(560)	-	-	+(460)	19.6.91
	If69	+(560)	-	+(470)	-	+(520)	20.6.91
	If75	+(520)	-	+(470)	+(600)	-	21.6.91
	If76	-	-	+(470)	+(600)	-	21.6.91

ND: Not done; + indicates the presence of an allele, - indicates that an allele was not detected. Figures in brackets are the allele sizes in base pairs

7.4. 2. Variations in allele frequencies of MSP-1 and MSP-2 within the different houses.

All three sequence variants of MSP-1 (K1-, MAD20- & RO33-type) and both of MSP-2 (IC1- & FC27-type) were found in the different households. To investigate the possibility of spatial clustering of the parasites within the hamlet, the observed allele frequencies (classified by sequence only) were compared to those of the whole village. No significant differences were seen ($G=1.3$; $P>0.3$)(Table 29). The MSP-1 alleles in each household were also compared to the whole village. Household 8003, 8004 and 8006 showed no significant differences from the rest of the village, however, significant differences in the alleles of household 8001 ($0.05>P>.02$) were found (Table 29).

The variants of MSP-2 showed no obvious significant differences within the households $P>0.7$ (table 30), or between the hamlet and the whole village $P>0.5$ (data not shown).

Table 29. Comparison of allele frequencies of the MSP-1 gene between Kininina hamlet and Michenga village, and between individual households in Kininina and Michenga village.

	K1 Obs (Exp)	Mad20 Obs (Exp)	RO33 Obs (Exp)	G value P value
Kininina	42 (38.53)	17 (18.73)	17 (18.73)	$G=6.5$
Michenga	30 (33.46)	18 (16.26)	18 (16.26)	$P>0.3$
8001/1	12 (10.50)	9 (6.75)	1 (4.750)	$G=6.5093$
Michenga	30 (31.50)	18 (20.25)	18 (14.25)	$0.05>P>.02$
8003/1-3	4 (4.474)	3 (2.76)	3 (2.763)	$G=0.105$
Michenga	30 (29.52)	18 (18.24)	18 (18.24)	$P>0.9$
8004/1-4	15 (12.00)	4 (5.867)	5 (6.133)	$G=2.15$
Michenga	30 (33.00)	18 (16.133)	18 (16.87)	$P>0.30$
8006/1-4	11 (9.535)	1 (4.419)	8 (6.05)	$G=5.6648$
Michenga	30 (31.465)	18 (14.581)	18 (19.95)	$0.1>P>0.05$

For each set of data $df=2$

Table 30. Comparison of allele frequencies of MSP-2 within households of Kininina.

house number	FC2	IC1	Total
	Obs (Exp)	Obs (Exp)	
8001/1	11 (10.84)	7 (7.15)	18
8003/1-3	4 (3.61)	2 (2.38)	6
8004/1-4	10 (12.05)	10 (7.94)	20
8006/1-4	11 (9.64)	5 (6.35)	16

G=1.47, df=3, P>0.7

7.4.3 Parasites infecting individuals in each household.

Parasites infecting the inhabitants of these houses although collected on a single day, were found to be very diverse. Isolates in each household had a different combination of alleles of the two genes. Multiclonal infections were very common. All infected individuals in house 8001/1 and 8003/1-3, five of eight in house 8004/1-4 and three of four in house 8006/1-4 had multiclonal infections (Table 31), which were found to be high (85%) among infected people in the whole village (chapter 6).

Table 31. MSP-1 & MSP-2 of isolates collected on the 21st June 1991, from infected individuals in four households in Kininina, Tanzania.

house number	Isolate number	MSP-1			MSP-2	
		K1	MAD20	RO33	IC1	FC27
8001/1-2	IfB2	+(520) -	+(470) +	-	-	+(600)
	IfB3	+(520)	+(520)	-	+(520)	+(480)
	IfB4	+(540)	-	+(470)	+(650)	+(480)
8003/1-3	IfB10	+(410)	+(470)	+(470)	+(600)	+(520)
	IfB11	+(470)	+(560)	-	+(520)	+(520)
8004/1-4	IfB13	-	+(560)	+(470)	-	+(580)
	IfB14	+(470)	-	+(470)	+(520)	+(520)
	IfB16	-	-	+(490)	+(650; 520)	+(600)
	IfB17	ND	ND	ND	-	+(520)
	IfB18	+(470)	-	-	+(520)	+(460)
	IfB20	+(470)	-	-	+(520)	-
	IfB22	+(580)	+(580)	+(470)	+(520)	+(600)
	IfB23	+(560)	-	+(470)	+(650)	+(520)
8006/1-4	IfB26	+(470)	-	-	+(650)	+(520)
	IfB27	+(540; 470)	-	-	+(650)	+(480)
	IfB28	+(580)	+(500)	+(470)	+(520)	+(520)
	IfB29	-	-	+(490)	ND	ND

ND: Not done; + presence of allele, - indicates absence of allele. Figures in brackets allele sizes in base pairs

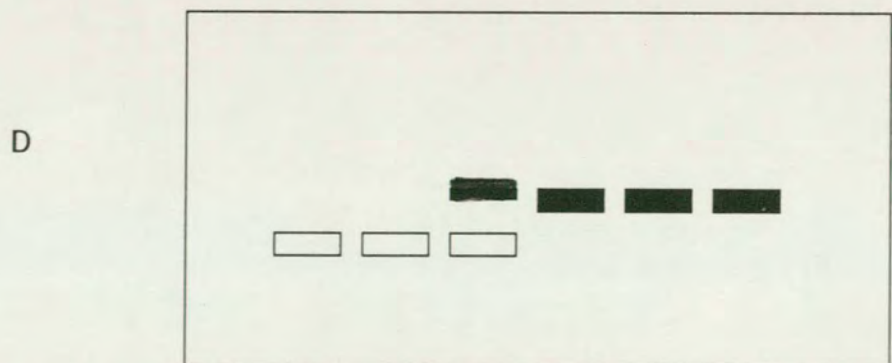
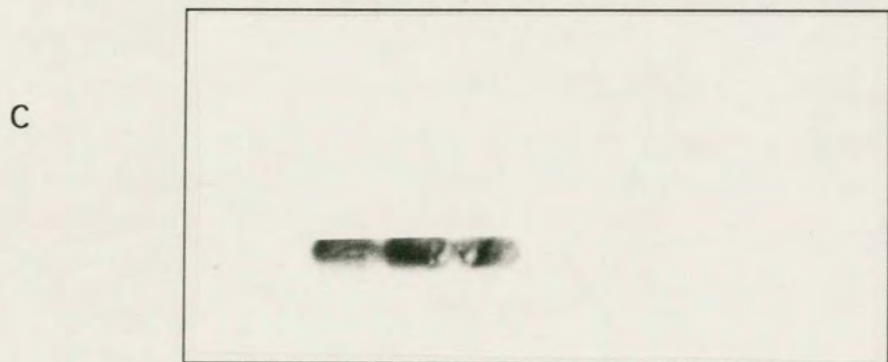
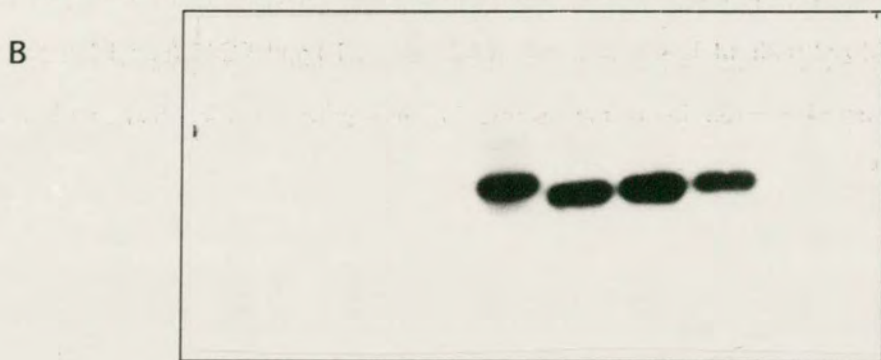
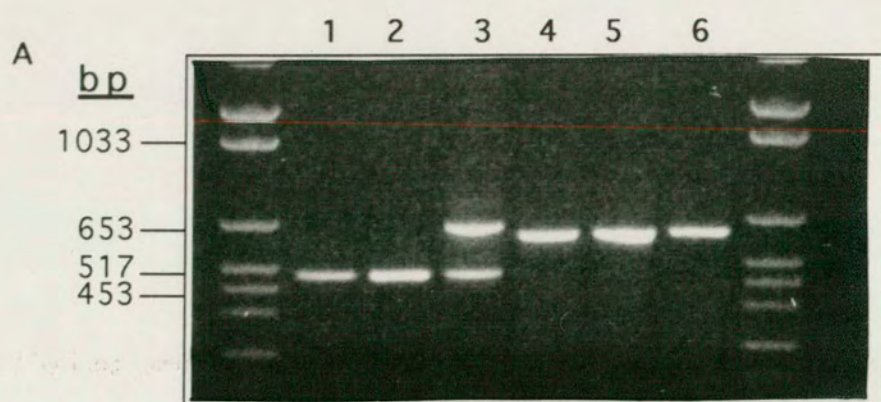
7.4.4 Comparison of parasites in people, parasites acquired by fed mosquitoes, and oocysts of a single hut (8001/1).

The abdomens of 12 blood fed mosquitoes were collected from hut 8001/1. Ten of these 12 gave a positive PCR product with one or both genes. Six were typed for the alleles of MSP-1 and/or MSP-2 using allele-specific probes (Table 32). Four of these 6 were found to have acquired blood meals with more than one clone, with different alleles of both genes (Figure 26).

The parasites of freshly fed mosquitoes, those collected from the blood of infected people and the oocysts collected from the same hut shared some alleles of each gene (Figure 27). However the parasites in the blood meals of the mosquitoes detected on the 24th & the 25th June, were different

Figure 26.

Alleles of MSP-2 gene of *P.falciparum* extracted from blood meals acquired by fed mosquitoes *A. gambiae* & *A. funestus*. Following electrophoresis (panel A), blots were hybridized with allele-specific probes IC1 (panel B) and FC27 (panel C); panel D shows a schematic interpretation of the results. Note mosquito in track 3 had acquired a blood meal that contained parasites of mixed genotypes.



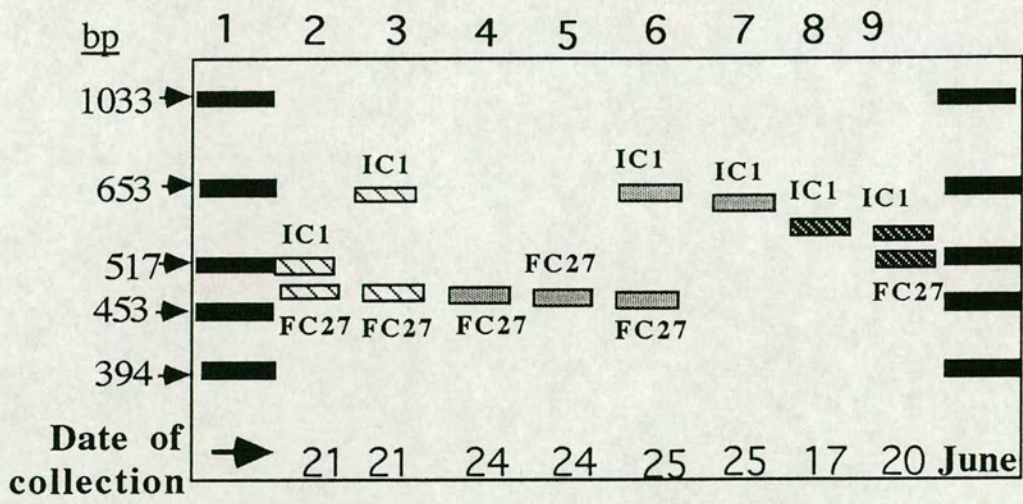
FC27 IC1




Figure 27.

Schematic illustration of the MSP-2 alleles of parasites infecting people, parasites acquired by fed mosquitoes and oocysts from mosquitoes collected from hut number 8001/1. Alleles were classified by size and sequence of PCR-amplified fragments.

*to be
was allelic
types detected?
See Table 32*

Household No. 8001/1



-  Asexual parasites
-  Fed mosquitoes
-  Oocysts

from the parasites collected from the inhabitants on the 21st June. The mosquitoes collected on the 19th June developed oocysts with different genotypes from those in the inhabitants collected on the 21st June.

Table 32 MSP-1 & MSP-2 of parasites extracted from fed mosquitoes collected from household (8001/1) in Kininina.

Mosquito number	coll. Date	MSP-1			MSP-2	
		K1	MAD20	RO33	IC1	FC27
F4	24.6.91	+(540)	-	+(470)	-	+(480)
F5	"	+(560)	+(560)	-	-	+(480)
F6	"	ND	ND	ND	+(650)	+(480)
F9	25.6.91	ND	ND	ND	+(630)	-
F10	"	ND	ND	ND	+(630)	-
F12	"	+	-	+(470)	+(630)	-

ND: Not done; + presence of allele, - absence of allele, figures in brackets are allele sizes in base pairs

7.5 Discussion.

Fewer alleles of MSP-1 and MSP-2 were found at the household level in Michenga compared to the whole village. 17 alleles of MSP-1 and 24 of MSP-2 were detected in the village, while there were only 8 and 11 respectively at the household level (Table 28). A smaller gene pool has been observed previously in smaller *P. falciparum* communities compared to larger ones (Conway & MacBride, 1991). However, there was no evidence of genotype clustering in any of the studied households. Each mosquito collected in these households was found to be infected with oocysts of different genotypes, and the inhabitants had parasites which could be distinguished by one or both genes. Thus, spatially structured parasite populations within which mating could be restricted, even to clonal propagation, as suggested by Day *et al.* (1992) was not seen in these houses.

The extensive polymorphism observed in each household is not surprising in such a highly endemic situation, where each unprotected individual can receive an annual average of 548 infective bites (Lyimo, 1993). Mosquito densities in these houses were exceptionally high

compared to the rest of the village and around 15% of them were found to be infected in the present study. Thus, the observed level of polymorphism is a consequence of the intensity of transmission and sexual reproduction between these parasites (chapter 6). The presence of homozygous and heterozygous oocysts showed that self- as well as cross-mating was occurring in all households. The homozygous oocysts (zygotes) in each household possessed different genotypes. Thus, even at such a local level, the observed self-mating does not indicate "clonality" (Tibayrenc *et al.*, 1990).

The study of individual oocysts has enabled a more precise determination of allele frequencies and genotypes within each household than is possible by studying blood forms alone. These households are dispersed within a rice farm, approximately 1 km apart. There was no significant difference between the allele frequencies of the two genes in Kininina hamlet, in Michenga village and within each household of Kininina hamlet. However, parasites of one household (8001) were found to have distinguishable allele frequencies of the MSP-1 gene. This might have happened because of the small sample sizes involved. Alternatively it might suggest some degree of isolation of this house. If so, the parasites might be partially isolated due to a patchy distribution of the mosquitoes.

In natural parasite populations any spatial clustering and partial isolation of parasites may have some epidemiological consequences. The mosquito vectors *A. gambiae* and *A. funestus* are predominantly anthropophilic and spend much of their adult life resting inside houses (Gillies, 1988). Recently emerged mosquitoes feed preferentially in houses near the breeding site where they have emerged (Gillies, 1988). Greater variation in mosquito densities between houses has been observed in Namawalla village in this region (Charlwood *et al.*, in prep). Thus, variation in the proximity of houses to breeding sites in the village could result in partial isolation of parasites to a particular household, within which gametocytes are acquired by mosquitoes at random from the inhabitants. However, such parasites are clearly not completely isolated, since many alleles are shared between them and the rest of the village.

Geographical variations in allele frequencies of particular genes of *P. falciparum* have been observed between parasites of different countries (Creasey *et al.*, 1990) which are probably due to barriers in movement of people and/or mosquitoes between different countries. Such variations have also been suggested to occur between villages widely isolated in a single region (Forsyth *et al.*, 1989). Using mark-release-recapture experiments, Charlwood *et al.* (1986) suggested that anophelene vectors return to the same village for subsequent blood meals. Such behaviour, if it occurs at a smaller geographical level, could confine the parasite to a limited geographical area.

This study has shown that in Michenga village random mating happens among parasites "substructured" in people, and free gene exchange occurs between parasites in different households. Although some indications for partial isolation of some parasites have been observed these parasites are not completely isolated and share alleles with other parasites in the village. For a better understanding of the transmission dynamics of *P. falciparum* such a molecular epidemiological approach should be considered to define the differentiation and structure in nature. It is therefore important to examine whether there is any correlation between geographic distribution pattern of alleles at several unlinked loci and quantify these differences if it does occur.

Chapter 8. Comparison of genetic diversity of *Plasmodium falciparum* in villages in Tanzania and Sudan.

8.1. Summary

The allelic diversity of MSP-1 and MSP-2 has been compared between Asar village in Sudan and Michenga village in Tanzania. Malaria in Michenga village of Tanzania is holo-endemic and transmission is throughout the year; in Asar village of Sudan, by contrast, malaria is meso-endemic and transmission is limited to 12-14 weeks. In Michenga, the numbers of alleles at both loci were greater than in Asar. The high degree of genetic polymorphism in Michenga is associated with a higher frequency of multiclonal infection in individual hosts than in Asar.

8.2. Study areas, subjects and methods used

The details of Asar village and the parasites studied have been given in section 2.1.1. For the purpose of this comparison, the 43 *P. falciparum* isolates collected from Asar village in the transmission season of 1991 were compared to the 53 samples collected in the same year from Michenga village (section 2.3.2). Isolates from the two villages were examined as described in previous chapters.

Estimation of both entomological inoculation rates (EIR) and parasite rates in Asar were carried out during the transmission season in 1990. The EIR in Asar was measured by a series of spray catches and indoor light trap catches, carried out in 5 to 10 houses every day over a period of 30 days. All mosquitoes were dissected and examined for sporozoites. A cross-sectional survey of parasite rates was carried out among 374 school children aged 7 to 15 years in October 1990. In Michenga, Tanzania the EIR was measured by Lyimo (1993).

Genomic DNA of all isolates collected in both Asar and Michenga was extracted as described (section 2.6.2.).

Alleles of the MSP-1 and the MSP-2 genes were determined using PCR (section 2.6.4.) and allele-specific oligonucleotide probes (section 2.6.6.).

8.3 Results

8.3.1 Comparison of parasite rates and entomological inoculation rates between Asar and Michenga.

(i) Parasite rates.

In Asar four (2%) out of 374 school children were found to have *P. falciparum*. Three of these had gametocytes and one had asexual forms. In Michenga by contrast 57 of 76 (75%) asymptomatic individuals of all ages were found to have *P. falciparum* infections.

(ii) Entomological inoculation rate (EIR).

A total of 157 rooms in Asar sampled by spray catch yielded 97 female *An.arabiensis*. Assuming that all females had fed on occupants of the rooms in which they were found, and that there was an average of 2 people per room, it can be calculated that the man biting rate was $97/(157 \times 2) = 0.31$ bites/person/night during the sampling period. Out of 97 mosquitoes dissected, 2 were found to be infected with sporozoites. The sporozoite rate was thus estimated to be 2.06%. Thus, while the sample size was small, these estimates imply an entomological inoculation rate (EIR) of 0.0064 (95% c.i. 0.00077 to 0.023) infective bites per person/night. Assuming that the transmission is confined to 90 days, an average of 0.57 (95% c.i. 0.03 to 0.92) infective bites/person/year is estimated.

Estimates of sporozoite rate and EIR in Michenga village were made by Lyimo (1993). Mosquitoes (*An.gambiae* and *An.funestus*) positive for sporozoites were found throughout the year

with a peak from June to December. The mean annual EIR for the area was estimated to be 548 infective bites/ person/year.

8.3.2 Polymorphism of MSP-1 and MSP-2 in Michenga.

41 of the 56 isolates collected from Michenga were successfully typed for both MSP-1 & MSP-2 genes 12 were typed for one or the other gene, and 3 did not give a PCR product for either gene.

PCR amplified fragments of each gene were sorted first according to the size of the PCR amplified fragments and then according to sequence following hybridization of both with allele-specific probes (section 2.6.6.). Both genes were found to be highly polymorphic within the village. At least 17 different alleles of MSP-1, distinguishable by both size and sequence were identified (Figure 28a). Similarly, at least 23 different alleles of MSP-2 were detected (Figure 29a).

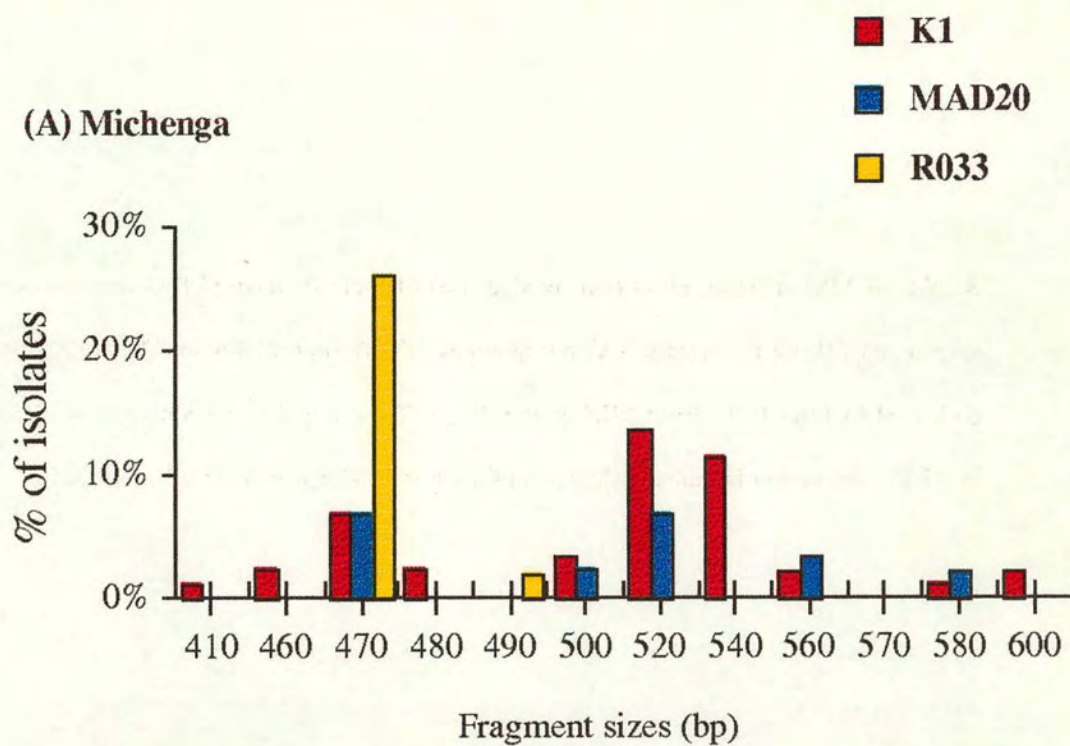
8.3.3 Polymorphism of MSP-1 & MSP-2 in Asar, Sudan.

Out of 485 febrile patients presented to the Asar clinic 121 (25%) were found to have *P. falciparum* and 3 (0.6%) had *P. vivax*. 43 *P. falciparum* isolates were collected for this study, which were typed for both genes. 9 alleles of MSP-1, and 13 alleles of MSP-2 were detected in the village (Figure 28b and Figure 29b).

Figure 28.

Alleles of MSP-1 gene, classified by size (bp) of their PCR-amplified fragments and sequence of these fragments. (A) percentages of different alleles in 53 *P.falciparum* isolates collected in June 1991 from Michenga village, Tanzania. (B) percentages of different alleles in 43 *P.falciparum* isolates collected in October 1991 from Asar village, Sudan.

(A) Michenga



(B) Asar

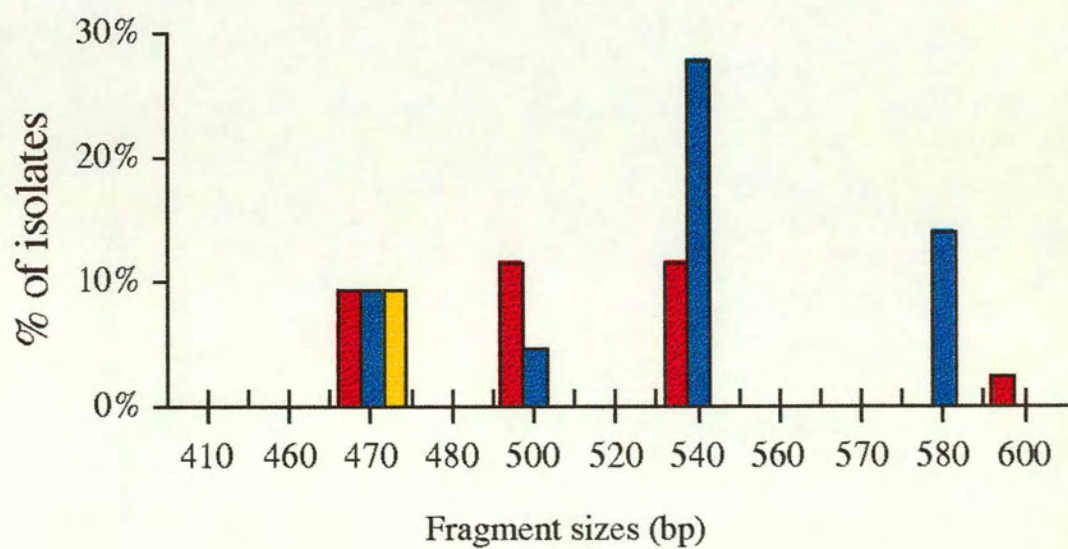
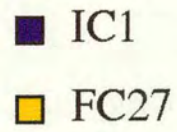
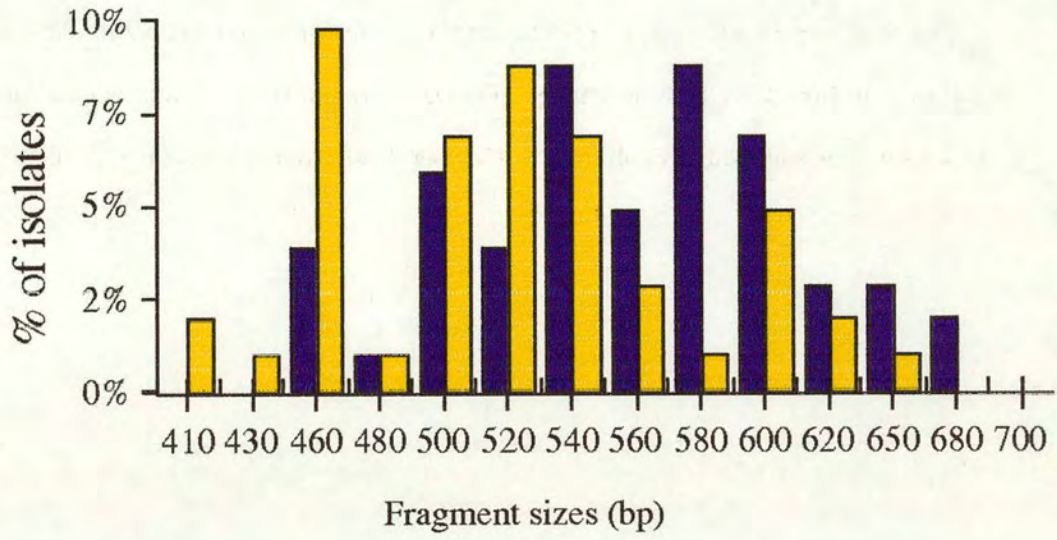


Figure 29.

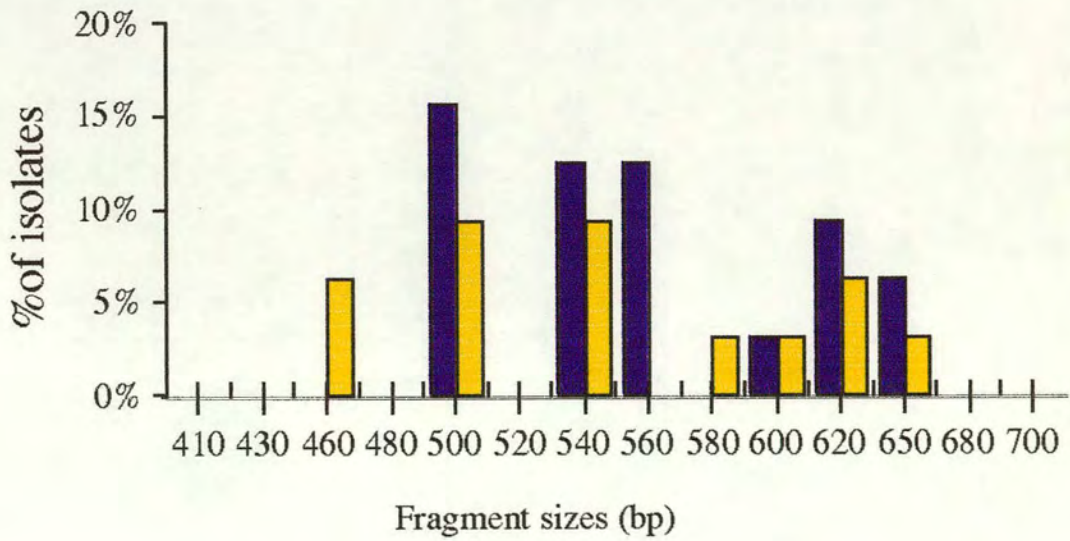
Alleles of MSP-2 gene, classified by size (bp) of their PCR-amplified fragments and sequence of these fragments. (A) percentages of different alleles in 53 *P.falciparum* isolates collected in June 1991 from Michenga village, Tanzania. (B) percentages of different alleles in 43 *P.falciparum* isolates collected in October 1991 from Asar village, Sudan.



(A) Michenga



(B) A sar



8.3.4 Comparison of MSP-1 and MSP-2 alleles in Michenga and Asar.

There is a higher level of polymorphism of both genes in Tanzania compared to Sudan. While 17 different alleles of MSP-1 were detected within Michenga, in a 3 day survey, only 9 alleles of this gene were seen in Asar, during a survey lasting for 13 days. All the 9 alleles seen in Sudan, except K1/500, were found in Tanzania but at clearly different frequencies. 9 alleles detected in Tanzania were not found in Sudan.

A similar pattern was seen with regard to MSP-2. At least 23 different alleles were detected in Michenga and only 13 in Asar. All MSP-2 alleles detected in Sudan were identified in Michenga, but with clearly different frequencies. 10 alleles detected in Michenga were not seen in Asar.

It is difficult to calculate allele frequencies in the malaria parasite population of a given community from the parasite genotypes in blood samples. The parasite load varies from one patient to another, and multiclonal infections with different proportions of genotypes commonly occur. Thus, in this study the numbers of the alleles of each gene in infected individuals was calculated in each village. whenever there was a multiclonal infection alleles were assumed to occur at equal proportions. Due to the limited size of the data, the frequencies of the alleles of the two genes were compared between the two villages according to their sequence only. In Michenga village, the MSP-1 sequence variants, K1, MAD20 and RO33, had frequencies of 44%, 26% and 30% respectively, while in Asar they were found at frequencies of 37%, 54% and 9% respectively (Figure 30a). The differences between the two communities were highly significant ($0.05 > P > 0.001$). Similarly the frequencies of the two sequence variants of MSP-2 in Michenga were found to be 50% and 50% for FC27 and IC1 respectively, while in Asar they were 35% and 65% respectively (Figure 30b), these differences are not significant ($0.2 > P > 0.1$).

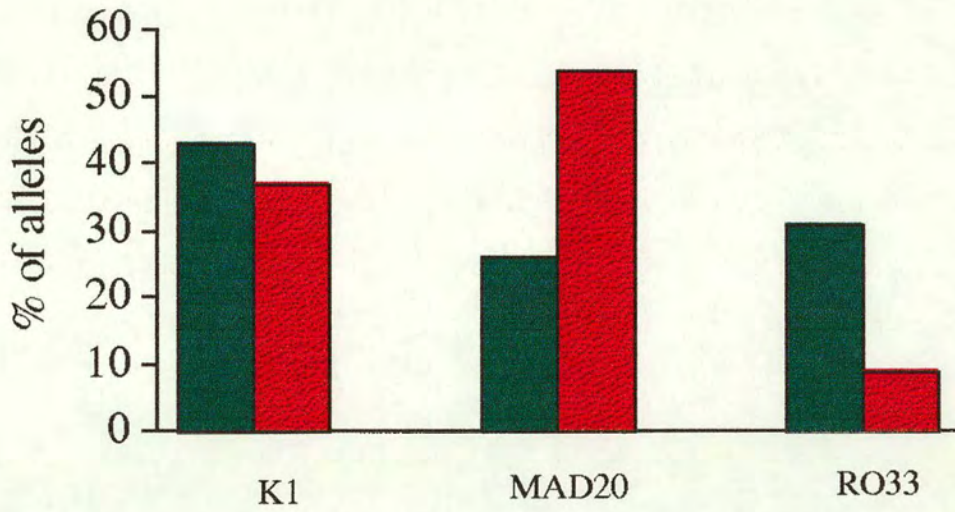
Figure 30.

Percentages of alleles of MSP-1 and MSP-2 genes, classified by the sequence of their PCR-amplified fragments only. (A) percentages of MSP-1 alleles (K1-, MAD20 and RO33-type) within 53 *P. falciparum* isolates collected in June 1991 from Michenga village, Tanzania, and 43 isolates collected in October 1991 from Asar village, Sudan. (B) percentages of MSP-2 alleles (IC1- and FC27-type) within the 53 isolates of Michenga village, and the 43 isolates of Asar village.

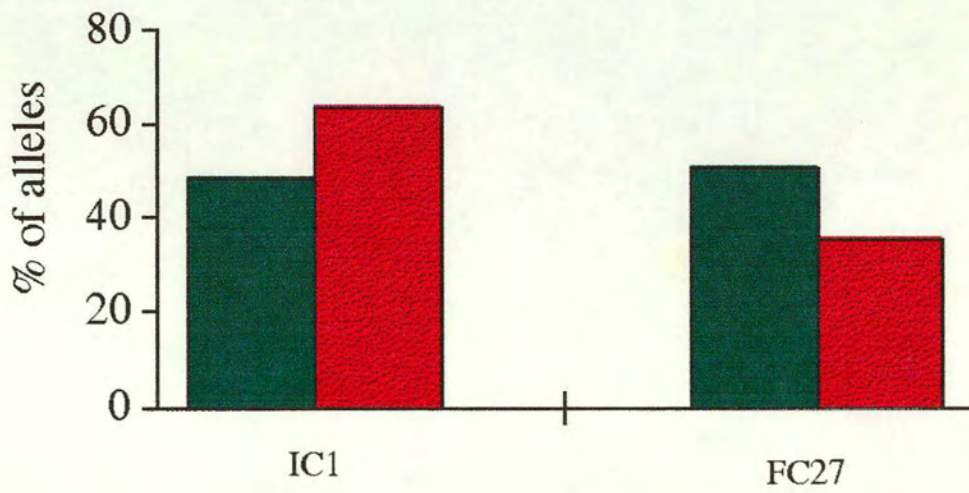
■ Tanzania

■ Sudan

(A) MSP-1



B. MSP-2



The frequency of multiclonal infection was found to be very high in Michenga. 45 out of 53 blood samples (85%) were found to contain mixtures of parasites, identified by the presence of two or more alleles of one or both genes (chapter 7). By contrast, in Asar village only 5 (12%) out of 43 patients were found to have multiclonal infections using these markers.

8.4 Discussion

The numbers of detectable alleles of the two antigen genes, MSP-1 and MSP-2, were significantly higher in Michenga village, Tanzania, than in Asar village, Sudan. This can probably be attributed to the following:- (1) Although the size of the population in the two villages is approximately similar, the high level of malaria endemicity in Michenga (Smith *et al.*, 1993, Lyimo 1993) allows a larger parasite population size and gene pool. The average EIR was estimated as approximately 1000 times higher and the parasite rate approximately 40 times larger in Michenga than Asar. (2) In an area with a high level of transmission such as The Gambia the parasite population was found to maintain its allele frequencies over many years (Conway *et al.*, 1992). In contrast, in the seasonal, small parasite population in Asar village changes in the alleles from one year to another were probably due to random genetic drift following a severe reduction in population size during the dry season (chapter 4). (3) It is possible that new alleles will be generated by intragenic recombination, as suggested for MSP-1 (Tanabe *et al.*, 1987; Conway *et al.*, 1991; Kerr, *et al.*, in prep) and MSP-2 (Marshall *et al.*, 1992). Such recombinant alleles are produced by intragenic recombination during meiosis of heterozygotes during mosquito transmission (Kerr *et al.*, in prep). Cross-mating between different genotypes has been found to occur frequently in Michenga (chapter 6).

Evidence based on *in vitro* cultures of *P. falciparum* have indicated that during mitotic division subtelomeric deletions which involving non coding repeat sequence and chromosomal rearrangements occur during mitotic division (reviewed by Janse, 1993). Not only non-coding repeat sequence but also genes may be subjected to such deletion (Kemp *et al.*, 1987). Thus,

despite the fact that the parasite population in Michenga, Tanzania, can maintain its alleles over time, new alleles might possibly be generated due to the high frequency of cross-mating. Long lasting infections in people might also result in genomic changes such as chromosomal deletions.

The frequency of multiclonal infections found in Michenga is probably the highest reported compared to other malaria endemic countries (Babiker *et al.*, 1991a; Conway, Greenwood & McBride, 1991; Kimura *et al.*, 1990; Snewin *et al.*, 1991). This can probably be explained by the intense transmission in Michenga where each individual can receive an average of 548 infective bites per year in a human population with a stable parasite rate of more than 80% (Lyimo, 1993; Smith *et al.*, 1993). The high parasite rate and inoculation rate leads to a high incidence rate (Molineaux, 1988), which presumably continuously accumulates new genotypes in the immune villagers. In The Gambia an average of 40%-50% of infected individuals harbour multiclonal infections (Conway *et al.*, 1991). Malaria there is classified as hyper-endemic and the average EIR has been estimated to be between 4-24 infective bites per person per year (Lindsay *et al.*, 1989). In Sudan, multiclonal infections using these genes was estimated in this study to occur at a frequency of 12%, a lower level than both Tanzania and The Gambia. Thus, the frequency of multiclonal infections seems to follow a gradation pattern consistent with the level of malaria endemicity estimated by the EIR and parasite rate in these three countries.

Based on the method used in this study the number of clones per person was estimated to be between 1-6 in Michenga. The average number of clones per person was estimated to be 3.2 in this village (chapter 6). In Sudan it was estimated to be between 1 to 2. In The Gambia using different methods it was estimated to be between 1 to 4 with an average of 2 (Carter and McGregor, 1975; Conway, Greenwood & McBride, 1991). The intensity and endemicity of malaria in The Gambia is lower than in Tanzania and higher than Sudan. This suggests that possibly both the frequency and magnitude (number of clones per person) of multiclonal infection might be determined by the level of malaria endemicity as has previously proposed (Creasey *et al.*, 1990).

As in previous findings (Conway, Greenwood & McBride, 1991) there was no apparent correlation between the severity of the infection in a patient and the number of clones harboured. All the individuals studied in Michenga were asymptomatic, but 85% had multiclonal infection. In contrast, all the individuals of Asar were febrile at the time of sampling, but only 12% had more than one clone. In Asar transmission is thought to cease over the long dry season leaving the inhabitants without experience of malaria during this time. Moreover, approximately one in four people would expect an infective bite per transmission season, leaving more than half the population without exposure to malaria possibly for two seasons.

Variations in the frequencies of alleles identified by the sequence of the amplified region on each gene confirm the earlier observations of Creasey *et al.* (1990) that there is variation in gene frequencies between different countries. The K1 type was found at similar frequencies in both countries (Tanzania and Sudan), and similar frequencies were found in West Africa (Scherf *et al.*, 1991) and South America (Kimura *et al.*, 1990; Snewin *et al.*, 1991). However, the two other alleles, MAD20 and RO33, showed variation between different countries. The RO33 type was found to be the major form of the MSP-1 gene in South America (Kimura *et al.*, 1990; Snewin *et al.*, 1991) and West Africa (Scherf *et al.*, 1991). In contrast the frequency of this allele in East Africa (Sudan, Tanzania) is significantly lower. Similar variation between East and West Africa was observed with regard to the MAD20 allele; while it was frequent both in Tanzania and Sudan, it was infrequent in South America (Kimura *et al.*, 1990; Snewin *et al.*, 1991) and West Africa (Scherf *et al.*, 1991).

Variation between East and West Africa was also observed with regard to serotypes of the MSP-1, identified by monoclonal antibodies. The dimorphic domain of the molecule identified by epitopes 6.1, 7.3 etc, (blocks 6 to 17). was found to be very low in West Africa (Conway *et al.*, 1992) compared to East Africa (Sudan). The alternative dimorphic domain, identified by Mabs 9.2, 9.7 and 10.3 was found at a high frequency in East Africa (Babiker *et al.*, 1991) compared to West Africa.

In conclusion this study has suggested that the genetic structure of natural populations is affected by the level of malaria endemicity. In areas with high malaria endemicity parasites are more polymorphic compared to areas with low endemicity. The high endemicity is also associated with high prevalence of multiclonal infection, and in such situations mosquito vectors can be expected to acquire blood meals containing gametocytes of more than one genotype frequently. Thus in highly endemic areas, cross-fertilization and recombination are likely to occur frequently to generate new genotypes, and possibly new alleles, compared to areas of low endemicity.

Chapter 9. General discussion and conclusions.

This chapter brings together the main points in the discussion of previous chapters, to take an overall view of the relevance of genetic studies to problems of control.

(1) Diversity in natural *P. falciparum* populations

(i) Technical developments in studying allelic polymorphisms

Conventional biochemical markers such as enzymes and 2D-PAGE protein have been used successfully for studying variations between clones and wild isolates of *P. falciparum* (Walliker, 1985; Fenton *et al.*, 1985; Creasey *et al.*, 1990). However, these techniques are limited by the need to establish wild isolates in culture (Trager and Jensen, 1976; Haynes *et al.*, 1976) which is usually a laborious and time consuming procedure. Thus, large scale surveys of natural parasites to address questions of genetic structure and spatial structuring of *P. falciparum* populations are not easily approached using these methods. Recent developments of PCR-based techniques to type parasites obtained from fingerprick blood samples (Foley, Ranford-Carwright & Babiker, 1992), parasites acquired by fed mosquitoes and individual oocysts (Ranford-Cartwright *et al.*, 1991) have made it possible to address these questions more easily. Genes containing variable numbers of tandem repeats (VNTR) as those shown in human DNA (Jeffreys *et al.*, 1990), can be analysed based on their size differences following PCR amplification, and their sequence type determined using radio-labelled probes (Kimura *et al.*, 1990). In this thesis, non-radioactive labelled probes were successfully used to study polymorphism of MSP-1 and MSP-2; this approach makes a large scale survey of these genes feasible. This technique can also be transferred to laboratories in developing countries because radio-active substances are not required. VNTR-type polymorphisms have attracted several workers to screen parasite populations in this way addressing some genetic and

epidemiological questions (e.g. Scherf *et al.*, 1990; Kimura *et al.*, 1990; Jongwutiwes *et al.*, 1992; Thomas *et al.*, 1990; Borre *et al.*, 1991; Arnot *et al.*, 1993).

(ii) Heterogeneity of natural populations

This study and others have shown clearly that members (clones) of natural *P. falciparum* population are very polymorphic, and coexist in communities of their human hosts. No evidence of over-representation of any genotype occurs within parasites of (Asar) Sudan (chapter 3) or (Michenga) Tanzania (chapters 6 and 7). The two villages are in areas representing the two extremes of transmission intensity; nevertheless, the parasite population in each village is remarkably polymorphic. The extent of this polymorphism is noticeable even at the level of the household within each of the studied villages. Individuals in two households in Asar village and four households in Michenga from whom parasites were collected on the same day, each had parasites of different genotypes. Allelic polymorphism of *P. falciparum* genes has been found to be a major feature of natural parasite populations in many endemic areas, The Gambia (Carter and MaGregor 1975; Conway & McBride 1991), Thailand, Zimbabwe and Brazil (Thaithong *et al.*, 1989; Creasey *et al.*, 1990), Senegal (Scherf *et al.* 1990), Colombia (Snwein *et al.*, 1991) and Papua New Guinea (Day *et al.*, 1992, Marshall *et al.*, 1994).

The mechanisms by which such allelic polymorphisms are generated and maintained is not fully understood. The predominance of non-synonymous base substitutions in non-repeat regions appears to be a general feature of *Plasmodium* genes (Arnot, 1989). For antigen genes, this has been interpreted as evidence of immune selection creating diversity in T and B cell epitopes (McCutchan *et al.*, 1989; Lockyer *et al.*, 1989), although an alternative view is that such mutations may be a general feature of the *Plasmodium* genome rather than a consequence of phenotypic selection (Arnot, 1989). An important cause of allelic polymorphism in nature is probably recombination events such as unequal crossing-over which can lead to the spread of mutations from one repeat to another. For some genes, such a process could generate novel

alleles of some genes, e.g S-antigens (Cowman *et al.*, 1985). In addition, novel alleles can be generated by intragenic recombination between two parental alleles (Tanabe *et al.*, 1987; Conway *et al.*, 1991; Jongwutiwes *et al.*, 1991; Kerr *et al.*, submitted for publication).

As most polymorphic antigens studied so far are located on different chromosomes, novel genotypes can be created by independent assortment of chromosomes, as well as by crossing-over following meiosis (Walliker *et al.*, 1987; Walker-Jonah, *et al.*, 1992). In this thesis, solid evidence has been obtained about the significance of these mechanisms in generating diversity in nature.

(iii) Heterogeneity of responses to antimalarials

One of the major epidemiological features of *P. falciparum* malaria in both Asar and Michenga is resistance to some antimalarials including chloroquine (Huber *et al.*, 1993; Babiker *et al.*, 1991b), which is widely used in both countries. In Asar, the parasites have been found to be very diverse with regard to their response to chloroquine and pyrimethamine. The use of genetically pure clones of these isolates permitted clearer differentiation of some characteristics of the drug resistant *P. falciparum* in the village. Clones exhibited different levels of response to chloroquine, low, intermediate and high level resistance co-existing in the parasite population. Similarly clones with sensitive and resistant responses to pyrimethamine, were found. Since clones with different combinations of responses to chloroquine and pyrimethamine coexist in the village, it is clear that gene(s) conferring resistance to both drugs are at different loci. It is certainly possible that sexual recombination during mosquito transmission could bring genes conferring resistance to both drugs together and generate multidrug resistance, a phenomenon which was not common in Sudan. Curtis and Otoo (1986) have hypothesised that in randomly mating parasite populations resistance to two drugs controlled by unlinked genes develop slowly, when the resistance genes are rare and there is no selection favouring these genes. According to our findings in Asar village, the parasite

population appears potentially interbreeding. However, resistance to chloroquine and pyrimethamine is frequent. Thus, it might be better to use a combination of the two drugs for treatment of *P. falciparum* in other parts of the country where resistance to both genes is rare (western Sudan).

In-vivo, clinically, resistance to chloroquine can be classified into three types R1, R11 and R111 (Bruce-chwatt, 1986), and of these the R1 type is indistinguishable from reinfections with new parasites. We took advantage of the high resolution capacity of the PCR and sequence-specific probing techniques to compare the MSP-1 and the MSP-2 genes of primary and suspected recrudescence parasites in Asar village. No two isolates in each of three years were found to share the same combinations of alleles at the two loci, but the recrudescence parasites were identical to their primary parasites, at least at these loci. Such an approach may provide a basis for a diagnostic tool to discriminate between reinfections and genuine recrudescence of an R1 chloroquine resistance type (Babiker *et al.*, 1994).

(iv) Multiclonal infections

A clear feature of natural populations of *P. falciparum* is sub-structuring of parasites in infected people. Frequently more than one genotype occurs in infected individuals. The frequency of such multiclonal infections was found to be high in many endemic countries (Babiker *et al.*, 1991a; Conway Greenwood & McBride, 1991; Kimura *et al.*, 1990; Snewin *et al.*, 1991). It has been considered as a normal feature of malaria epidemiology and has been incorporated into many mathematical models aiming to simulate natural situations (Deitz, Molineaux & Thomas, 1974). The phenomenon could be explained by superinfections where the transmission level is high. Alternatively it could be explained by inoculation of non-identical clones from a single infected bite. Such a mosquito may contain sporozoites resulting from cross-fertilization between unlike genotypes, or unrelated sporozoites which had developed from different oocysts.

Both the frequency and magnitude of this substructuring of parasite in people seems to correlate with the degree of transmission. In Tanzania both were found to be higher than reported before from other endemic areas with lower levels of transmission. In Michenga village an unprotected individual can receive up to 100 infectious bites per night (Lyimo, 1993). This study has found that the number of clones per person in this village ranged from 1 to 6 (the upper limit of detection of the method used) with an average of 3.5. In The Gambia it ranges from 1 to 4 with an average of 2 clones per patient (Carter and Voller, 1975; Conway, Greenwood & McBride, 1991), which is lower than we observed in Tanzania. In Thailand, where transmission is higher than in The Gambia, 7 clones have been detected in one individual (Thaithong *et al.*, 1984).

The frequency and magnitude of multiclonal infections has a significant impact on malaria epidemiology. Multiclonal infection is a pre-requisite for cross-mating and recombination, for generation of new genotypes and possibly new alleles within a given parasite population. Most of the reported multiclonal infections have been detected by examination of the asexual forms of the parasite. However, it has been suggested that all merozoites might have a capacity to produce gametocytes (Bruce *et al.*, 1990). In chapter 6 it could be deduced that all the asexual genotypes in Michenga were transmissible to mosquitoes. There was hardly any difference between the frequencies of the alleles detected in the infected people and in the mosquitoes (oocysts). Gametocyte half-life has been suggested to be long (Smalley & Sinden, 1977). Thus, in a hyper-endemic situation, mating can readily occur between already existing genotypes and new ones resulting from the continuity of infective bites.

(2) Population genetic structure

Laboratory studies have clearly demonstrated that gametocytes of both *P. falciparum* and *P. vivax* undergo both self- and cross-fertilization in the mosquito vector (Ranford-Cartwright *et al.*, 1993; Rosenberg *et al.*, 1992). In this study, analysis of individual oocysts in wild-caught mosquitoes has enabled the direct genotyping of the products of the diploid zygotes, and an assessment of frequencies of self- and cross-mating. Analysis of the data has confirmed that the *P. falciparum* parasites of Michenga village can be regarded as randomly interbreeding. The frequencies of oocyst genotypes did not depart from an expectation of linkage equilibrium between the studied genes. Thus, this work represents the first firm evidence of random mating in a natural population of *P. falciparum*.

Previous attempts to analyse mating patterns of natural *P. falciparum* populations have used data obtained from asexual blood forms, which are less definitive for allele and genotype frequencies. However, it has been concluded from such indirect evidence that random mating occurs in natural population of *P. falciparum* (Carter & McGregor, 1973; Conway & McBride, 1991), *P. chabaudi* (Beale *et al.*, 1978) and *P. vivax* (Joshi *et al.*, 1989). Recently an independent analysis of the results of the 29 isolates collected from Asar (chapter 3) by Maynard Smith *et al.* (1993) has come to a similar conclusion.

The model used in this thesis to describe the genetic structure of *P. falciparum* has taken into account the fact that natural malaria parasites are not regularly dispersed among their hosts. Individual contact between parasites depends entirely on their mosquito vectors. Thus, mating can only occur mainly between gametes from the same vertebrate host, except on the rare occasions when a single mosquito takes gametocytes from two infected people by interrupted feeding. Samples collected from these sub-populations can give rise to an apparent deficit of heterozygotes. However, chapter 6 has shown that the diploid genotype frequencies observed can be adequately explained by random mating between gametes from such a substructured parasite population. Such an approach may be of great help in analysing the population genetic

structure of parasites, which are known to disperse contagiously (with variance bigger than the mean) in their host. The dispersion of these parasites among their hosts often shows a very good agreement with the distribution predicted by negative binomial models (Fowler & Cohen, 1990).

The population genetic structure of protozoan parasites has been a topic of great debate, which is still continuing. It has been argued by Tibayrenc *et al.* (1990; 1991) that many parasitic protozoa, including *Plasmodium* have a clonal population structure. If so, this would have significant implications for the epidemiology and control of these parasites. Results obtained from the two villages studied in this work does not support this hypothesis. However these results have provided clear evidence that *P. falciparum* undergoes sexual reproduction in nature, a finding which may add to a better understanding of the biology of the parasite, the epidemiology of malaria and rational approach to control. With regard to control efforts based on anti-malarials, genetic studies have shown that responses to some anti-malarials are clearly genetically determined (Walliker *et al.*, 1987). For pyrimethamine, the gene associated with resistance (DHFR-TS) has been identified (Peterson *et al.*, 1990). The maintenance and spread of such polymorphisms among natural populations depends on the rate of genetic exchange within parasite populations. In a panmictic population, gene flow occurs readily, thus, the only possible obstacles being geographic and ecological factors. A mathematical model has predicted that, in absence of selection, resistance to two drugs determined by unlinked genes spreads more slowly when frequent recombination occurs than in its absence (Curtis & Otoo, 1986).

It is unknown whether allelic polymorphism of the merozoite surface proteins MSP-1 or MSP-2 will be an obstacle to vaccine development. Polymorphism is a potential problem only if protective immune responses are directed against polymorphic domains of either protein, rather than against conserved epitopes. Polymorphic regions of the MSP-1 have been suggested to be immunogenic (Tolle *et al.*, 1993). However, recently evidence has been obtained that the conserved C-terminal of MSP-1 of *P. yoelii* protects mice against challenge infection (Ling,

Ogun & Holder, 1994). If this is confirmed then the generation of novel alleles of the antigen during sexual reproduction may be of no importance to the efficacy of a vaccine based on this region of the molecule. However, recently human immune responses to defined fragments of MSP-1 and MSP-2, have been investigated using recombinant molecules of the two antigens, to determine to which region of the proteins such responses are targeted. The dimorphic region of the MSP-1 was suggested to be a target of a humoral immune response in individuals in endemic areas (Fruh *et al.*, 1991). This immune response was found to be short-lived and dropped significantly at the end of the dry season. Thus, antigenic polymorphism in this region would be sufficient for repeated infection in host. Similarly, humoral immune responses to MSP-2 appear to be directed against polymorphic rather than the conserved regions of this antigen (Taylor *et al.*, 1993). So, given the evidence that sexual recombination is frequent in nature, incorporation of all the different alleles of such immunogenic antigens in a multivalent vaccine may need to be considered.

The causes of a new episode of malaria illness in endemic countries are not understood, seasonality of morbidity has been observed in endemic countries where malaria is stable and transmission is perennial (Greenwood *et al.*, 1987; Snow *et al.*, 1993). Although one possible explanation for such a pattern is infection with a novel "strain" of *P. falciparum*, to which the patient has not been previously exposed (Lines and Armstrong, 1992). It has been suggested that the response to an immunogenic antigen is allele specific and it is short-lived (Fruh *et al.*, 1991). The seasonality of the peaks of transmission is associated with high vector density, and this may be accompanied by frequent mating between the existing genotypes to generate novel parasites which are not been recognised by the immune response. However, in this context it should be noted that the recrudescence R1 chloroquine resistant parasites, detected in six patients in Asar, caused febrile episodes on each occasion they presented at the clinic. Thus in these instances the fever was not caused by infection with a new strain. Nevertheless this hypothesis needs systematic analysis of seasonality of malaria morbidity and the frequency of cross-mating in areas where transmission is high and people remain infected through the year.

(3) Spatial structure of natural parasites

Despite the fact that natural *P. falciparum* are diverse and sexually reproducing, allele frequencies of some genes vary in different countries (Creasey *et al.*, 1990). For a better understanding of the parasite transmission dynamics, the significance of these variations must be qualified and quantified. Such an approach may help to define the spatial structuring (units of transmission) of these parasites. Spatially related parasites can be regarded as sharing common alleles as a result of common ancestor. Thus, genes controlling response to drugs or protective immunity may be similar in a given area. The parasites of Asar, Sudan were found to have different frequencies of alleles of both genes, MSP-1 and MSP-2. These alleles were found to exhibit different frequencies in different countries in West Africa and South America (Scherf *et al.*, 1990; Snwein *et al.*, 1991). Thus, these variations are consistent with the geographical differentiation of *P. falciparum* populations (Creasey *et al.*, 1990). This is probably due to geographical barriers between the parasite populations as well as different selective forces. Among panmictic organisms gene flow can only be hindered by isolation or variations in selective forces. To assess whether parasites at local levels are substructured or homogeneously mixed, parasites in households in the Kininina hamlet of Michenga village, which are widely dispersed within farms, were examined. No evidence of variation in the frequencies of MSP-1 and MSP-2 alleles in different houses were observed, or between these alleles in the hamlet and the whole village. This suggested that gene flow occurs freely within parasites of this village. However, one household was found to be significantly different from the rest of the village. In this house the parasites might be partially isolated due to patchiness of the mosquitoes, probably due to proximity to a mosquito breeding site. Variation in mosquito density has been observed between different households in the village (Charwood *et al.*, in prep). Heterogeneity of transmission of malaria has been observed at different geographical levels (Molineaux & Gramiccia, 1980) and different households (Gama-Mendis *et al.*, 1989). The patchiness of the parasite population has been suggested to be advantageous to the parasite. Both the basic

reproduction rate and vectorial capacity have been suggested to be greater than under homogeneous mixing (Dye & Hasiberder, 1986). The observations presented in this study are preliminary, and a bigger scale study is recommended to quantify and qualify the spatial structure of *P. falciparum* in nature.

(4) Effect of malaria endemicity on parasite populations

This work has also investigated the possible impact of the pattern and level of transmission intensity on *P. falciparum* populations. First, in Asar village where malaria transmission is seasonal, significant fluctuations in allele frequencies from one year to another have been detected. The parasite population in the village may be subjected to a severe reduction in size during the long dry season which follows the end of rain. This pattern of transmission may be accompanied by fluctuation in gene frequencies as a result of random genetic drift. Nevertheless, the diversity observed over the studied period suggests that the annual resurgence of parasites following rains is accompanied by sexual recombination between the fewer genotypes that are expected to survive the dry season. Parasites collected from the early clinical cases of malaria in the village were found to be very diverse (chapter 4). In the Gambia, where malaria endemicity is higher, parasites were found to maintain their allele frequencies over time (Conway *et al.*, 1992). The effect of transmission on parasites in sub-Saharan Sudan may have some epidemiological significance. With regard to drug resistance, resistance controlled by more than one unlinked rare genes may take a long time to spread throughout the whole population.

Other possible effects of transmission intensity have been observed when parasites of Asar village of Sudan was compared to Michenga of Tanzania. The number of observed alleles of the two genes, MSP-1 and MSP-2, was bigger in Michenga compared to Asar despite the fact that the size of the two villages is approximately identical. This indicates that parasite

population and the gene pool is bigger in Michenga than in Asar. Both parasite rate and entomological inoculation rate are greater in Michenga.

This work has provided strong evidence that sexual reproduction takes place among natural populations of *P. falciparum*. Many of the genes studied so far are those which confer resistance to antimalarials, or induce immune responses. These genes are polymorphic, with many alleles coexisting in local parasite communities. Thus the capacity of the parasite to generate novel genotypes by cross-mating appears limitless. Sexual reproduction must be advantageous to this parasite, which has demonstrated an outstanding capacity to overcome all hurdles of control attempted so far. This must be surely taken into account when rational control measures based on chemotherapy or vaccination are contemplated.

References

- Al Tawil, N. and Akood, M. A. (1983) Response of falciparum malaria to a standard regimen of chloroquine in Khartoum province, Sudan. *East African Medical Journal* **60**: 663-668.
- Aley S. B., Barnwell J. W., Wendell D. and Howard R. J. (1984) Identification of parasite proteins in a membrane preparation enriched for surface membrane of erythrocytes infected with *Plasmodium knowlesi*. *Molecular & Biochemical Parasitology* **12**: 69-84.
- Alonso, P.L., Lindsay, S.W., Armstrong, J.R.M., Fegan, G., De Francisco, A., Hall, A.J., Shenton, F.C., Cham, K. & Greenwood, B.M. (1991) The effect of insecticide-treated bed nets on mortality of Gambian children. *Lancet* **337**: 1499-1515.
- Arnot, D. (1989) Malaria and the major histocompatibility complex. *Parasitology today* **5**(5): 138-146.
- Arnot, D.E. Roper, C. Bayoumi, R.A. (1993) Digital codes from hypervariable tandemly repeated DNA sequences in the *Plasmodium falciparum* circumsporozoite gene can genetically barcode isolates. *Molecular & Biochemical Parasitology* **61** :15-24
- Awad-El-Kariem, F.M., Miles M.A. and Warhurst, D.C. (1992) Chloroquine-resistant *Plasmodium falciparum* from the Sudan lack two mutations in the pf mdr-1 gene thought to be associated with chloroquine resistance. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86**: 587-589.
- Babiker, H. A, Creasy, A. M., Fenton, B., Bayoumi, R. A., Arnot, D. and Walliker, D. (1991a) Genetic Diversity of *Plasmodium falciparum* in a village in eastern Sudan: 1. Diversity of enzymes, 2D-PAGE proteins and antigens. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85**: 572-577
- Babiker, H. A, Creasy, A. M., Fenton, B., Bayoumi, R. A., Arnot, D. and Walliker, D. (1991b) Genetic Diversity of *Plasmodium falciparum* in a village in eastern Sudan: 2. Drug resistance, molecular karyotypes and the mdr1 genotype of recent isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85**: 578-583.
- Babiker, H. A, Ranford-Cartwright, L., Sultan, A., Satti, G. and Walliker, D. (1994) Genetic evidence that R1 chloroquine resistance of *Plasmodium falciparum* is caused by recrudescence of resistant parasites. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**: (in press).
- Bahr & Mikel (1972) The arrangement of DNA in the nucleus of rodent malaria parasites. *Proc. Helminthol. Soc. Wash.* **39** (special issue) 369-372.
- Bayoumi, R.L., Babiker, H.A., Ibrahiem, S.M., Chalib, H.W., Saeed, B.O., Khider, S., Elwasila, M.; and Karim, E.A. (1989) Spread of chloroquine resistant *Plasmodium falciparum* in Sudan. *Acta Tropica* **46**, 157-165.
- Bayoumi, R.L.; Creasey, A.M., Babiker, H.A., Carlton, J.M-R., Sultan, A., Satti, G., Sohal, A.W., Walliker, D. Jensen, J.B. & Arnot, D. (1993) Drug response and genetic characterization of *Plasmodium falciparum* clones recently isolated from a Sudanese village. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **87**: 454-458
- Beale, G.H., Carter, R. & Walliker, D. (1978) Genetics, In: Killick-Kendrick, R. and Peters, R. (eds), *Rodent Malaria*, Academic Press, London. pp 213-245.

- Bjorkman, A. & Philips-Howard, P.A. (1990) The epidemiology of drug resistant malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 84: 177-180.
- Blackman, M.J., Heidrich, H-G., Donachie, S., McBride, J.S., & Holder, A.A. (1990) A single fragment of malaria merozoite surface protein remains on parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *Journal of Experimental Medicine* 172: 379-382.
- Blackman, M.J., Whittle, H. & Holder, A. (1991) Processing of the *Plasmodium falciparum* major merozoite surface protein-1: identification of a 33-kilodalton secondary processing product which is shed prior to erythrocytic invasion. *Molecular & Biochemical Parasitology* 49:35-44
- Blackman, M.J., Chappel, J.A., Shai, S., Holder, A. (1993) A conserved parasite serine protease processes the *Plasmodium falciparum* merozoite surface protein-1. *Molecular & Biochemical Parasitology* 62:103-114
- Boreham, P.F.L, Lenehan, J.K., Boulzaguet, R., Storey, J., Ashkar, T.S., Nambiar, R. & Matsushima, T. (1979) Studies on multiple feeding by *Anopheles gambiae* s.i. in a Sudan savanna area of north Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 73: 418-423.
- Borre, M.B., Dziegiel, M., Hogh, B., Peterson, E., Rieneck, K., Riley, E., Meis, J.F., Aikawa, M., Nakaura, K., Harada, M., Wind, A., Jakobsen, P.H., Cowlam and, J., Jepsen, S., Axelsen, N.H. and Vuust, J. (1991) Primary structure and localization of a conserved immunogenic *Plasmodium falciparum* glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of vertebrate life cycle. *Molecular & Biochemical Parasitology* 49: 119-132
- Bruce, M., Alano, P., Duthie, S. and Carter, R. (1990) Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* 100: 191-200.
- Bruce-Chwatt, L.J. (1982) Imported malaria: an uninvited guest. *British Medical Bulletin* 38(2): 179-185
- Bruce-Chwatt, L.J. (1985) Essential Malariology, William Heinemann Medical Books Ltd., London.
- Bruce-Chwatt, L.J. Black, R.H., Canfield, C.J., Clyde, D.F. and Peter, W. (1986) In "Chemotherapy of Malaria" revised second edition. World Health Organisation, Geneva.
- Carlin, J. M., Vande-Was J. A., Jensen, J. B., Akood, M.A. S. (1894) African serum interence with the determination of chloroquine sensitivity in *Plasmodium falciparum*. *Zeit. Parasitenk* 70: 589-597.
- Carter, R. & McGregor, I.A. (1973) Enzyme variation in *Plasmodium falciparum* in The Gambia. *The Transactions of the Royal Society of Tropical Medicine and Hygiene* 67: 830-837
- Carter, R. & Voller, A.(1975) The distribution of enzyme variation in populations of *Plasmodium falciparum* in Africa. *The Transactions of the Royal Society of Tropical Medicine and Hygiene* 69: 371-376.
- Carter, R. (1978) Studies on enzyme variation in the murine malaria parasites *Plasmodium berghei*, *P.yoelii*, *P.vinckei*, and *P.chabaudi* by starch gel electrophoresis. *Parasitology*, 76: 241-267.

- Certa, U., Rotmann, D., Matile, H. & Rober-Liske, R. (1988) A naturally occurring gene encoding the major surface antigen precursor p190 of *Plasmodium falciparum* lacks tripeptide repeat. *EMBO Journal* 6: 4137-4142.
- Charlwood, D., Graves, P. & Birley, M. H. (1986) Capture-recapture studies of *Anopheles punctulatus* Donitz (Diptera: Culicidae) from Papua New Guinea. *Bulletin of Entomological Research* 76: 211-227.
- Charlwood, D.J., Smith, T., Kihonda, J., Billingsley, P.F. & Takken, W. Density independent feeding success in malaria vectors in Tanzania. (in prep)
- Church, G.M. & Gilbert, W. (1984) Genomic sequencing. *Proceedings of the National Academy of Science. USA*. 81: 1991-1995.
- Clark, J.T, Donachie, S., Anand, R., Wilson, C.F., Heidrich, H.,G., & McBride, J.S. (1989) 46-53 Kilodalton glycoprotein from surface of *Plasmodium falciparum* merozoites. *Molecular & Biochemical Parasitology* 32: 15-24
- Conway, D. J., Greenwood, B. M. & McBride, J. S.(1991) The epidemiology of multiple-clone *Plasmodium falciparum* infections in Gambian patients. *Parasitology* 103: 1-6.
- Conway, D. J. & McBride, J. S.(1991) Population genetics of *Plasmodium falciparum* within a malaria hyper-endemic area. *Parasitology* 103: 7-16.
- Conway, D. J., Rosario, V.E., Odoula, A., Salako, L., Greenwood, B. M. & McBride, J. S.(1991) Intragenic recombination and nonrandom associations between polymorphic domains of the major merozoite surface antigens. *Experimental Parasitology* 73:469-480.
- Conway, D. J., Greenwood, B. M., McBride, J. S. (1992) Longitudinal study of *Plasmodium falciparum* polymorphic antigens in a malaria-endemic population. *Infection and Immunity* 60: 1122-1127
- Corcoran, L.M., Forsyth, K.P., Bianco, A.E., Brown G.V. & Kemp, D.J. (1986) Chromosome size polymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite populations. *Cell* 44:87-95.
- Corcoran, L.M., Thompson, J.K., Walliker, D. & Kemp, D.J. (1988) Homologous recombination within subtelomeric repeat sequences generates chromosome size polymorphism in *Plasmodium falciparum*. *Cell* 53:807-813
- Cowman, A.F., Saint, B.R., Coppel, R.L., Brown, G.V., Anders, R. F. & Kemp, D. (1985) Conserved variable tandem repeats in two S-antigen genes of *Plasmodium falciparum*. *Cell* 40:775-783.
- Creasey, A. C., Fenton, B., Walker, A., Thaithong, S., Oliveira, S., Mutambu, S. & Walliker, D. (1990) Genetic diversity of *Plasmodium falciparum* shows geographical variation. *American Journal of Tropical Medicine and Hygiene* 42: 403-413.
- Curtis, C.F. & Otoo, L.N (1986). A simple model for the build up of resistance to mixture of anti-malarial drugs. *The Transactions of the Royal Society of Tropical Medicine and Hygiene* 80: 889-892.
- Curtis, C.F. (1993) Malaria control with vector as the target: strategy, options and research priorities. 11th Annual Joint Scientific Conference. Tanzania, N.I.M.R., Arusha, February 1993.

- Day, K.P., Koella, J.C., Nee, S., Gupta, S. & Read, A. (1992) Population genetics and dynamics of *Plasmodium falciparum*: an ecological view. *Parasitology* **104**: S35-S52.
- Daly, T.M., Burns, Jr., J.M. & Long, C.A. (1992) Comparison of the carboxy-terminal, cysteine-rich domain of the merozoite surface protein-1 from several strains of *Plasmodium yoelii*. *Molecular and Biochemical Parasitology* **28**:1-10
- Daly, T.M. & Long, C.A. (1993) A recombinant 15-Kilodalton carboxy-terminal fragment of *Plasmodium yoelii* 17XL merozoite surface protein 1 induces a protective immune response in mice. *Infection and Immunology* **61**:2462-2467.
- Deitz, K. Molineaux, L. & Thomas, A. (1974) A malaria model tested in the African savannah. *Bulletin of the World Health Organization* **50**:347-357.
- Deming, M.S., Gayibor, A., Murphy, K., Jones, T.S. & Kersa, T. (1989) Home treatment of febrile children with antimalarial drugs in Togo. *Bulletin of the World Health Organisation* **67**(6):695-700.
- Deleersnijder, W., Hendrix, D., Bendahman, N., Hanegreets, J., Brijs, L., HamersCasterman, C., & Hamers, R. (1990) Molecular cloning and sequence analysis of the gene encoding the major merozoite surface antigen of *Plasmodium chabaudi* IP-PCI. *Molecular and Biochemical Parasitology* **43**:1-10
- Dye, C. & Hasibeder, G. (1986) Population dynamics of mosquito-borne disease: effect of flies which bite some people more frequent than others. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 69-77.
- El Gadal, A. (1986) Malaria in the Sudan. In: Proceedings of the conference on Malaria in Africa, Bruck, A.A. (editor). Washington: American Institute of Biological Sciences, pp. 156-159.
- Endicott, J.A. & Ling V. (1989) The biochemistry of P-glycoprotein mediated multidrug resistance. *Annual Review of Biochemistry* **58**: 137-171.
- Epping, R.J., Goldstone, S.D., Ingram, L.T., Upcroft, J.A., Ramasamy, R., Cooper, J.A., Bushell, G.R. & Geysen, H.M (1988) An epitope recognised by inhibitory monoclonal antibodies that react with a 51-kilodalton merozoite surface antigen in *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **28**: 1-10
- Fenton, B., Walker, A. & Walliker, D. (1985) Protein variation in clones of *Plasmodium falciparum* detected by two dimensional electrophoresis. *Molecular and Biochemical Parasitology* **16**: 173-183.
- Fenton, B. Clark, J.T., Wilson, C.F., McBride, J.S. & Walliker, D. (1989) Polymorphism of a 35-48 kDa *Plasmodium falciparum* merozoite surface antigen. *Molecular and Biochemical Parasitology* **34**: 79-86.
- Fenton, B. and Walliker, D. (1990) Genetic analysis of polymorphic proteins of the human malaria parasite *Plasmodium falciparum*. *Genetical Research Cambridge* **55**: 81-86.
- Fenton, B., Clark, J.T., Anjam Khan, C.M., Robinson, J.V., Walliker, D., Ridley, R., Scaife, J.G. & McBride, J.S. (1991) Structural and antigenic polymorphism of the 35- to 48-Kilodalton merozoite surface antigen (MSA-2) of the malaria parasite *Plasmodium falciparum*. *Molecular and Cellular Biology* **11**, 963-971.
- Fogh, S., Jepsen, S. & Effersoe, P. (1979) Chloroquine resistant *Plasmodium falciparum* malaria in Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **73**: 228-229.

- Foley, M., Ranford-Cartwright, L.C. & Babiker, H.A. (1992). Rapid and simple method for isolating malarial DNA from fingerprick samples of blood. *Molecular and Biochemical Parasitology* **53**: 241-244.
- Foote, S.J., Thompson, J.K., Cowman, A.F. & Kemp, D.J., (1989) Amplification of the multi drug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell*, **57**:921-930.
- Foote, S.J., Kyle, D.E., Martin, R.K., Oduola, A.M.J., Forsyth, K., Kemp, D.J. & Cowman, A.F. (1990) Several alleles of the multidrug resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature* **345**: 255-258.
- Forsythe, K.P., Anders, R.F., Cattani, J.A. & Alpers, M.P. (1989) Small area variation in the prevalence of an S-antigen serotype of *Plasmodium falciparum* in villages of Madang, Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* **40**: 344-350.
- Fowler, J. & Cohen, L. (1990) Practical statistics for field biology. John Wiley & Sons, Chichester, New York, Brisbane, Toronto, Singapore.
- Freeman, R.R. & Holder A.A. (1983) Characteristics of protective response of BALB/c mice immunized with purified *Plasmodium yoelii* schizont antigen. *Clinical and Experimental Immunology* **54**:609
- Fruh, K., Doumbo, O., Muller, H-M., Koita, O., McBride, J., Crisanti, A., Toure, Y. & Bujard, H. (1991) Human antibody response to the major merozoite surface antigen of *Plasmodium falciparum* is strain specific and short-lived. *Infection and Immunity* **59**(4):1319-1324
- Gamage-Mendis, A.C., Carter, R., Mendis, Dezoysa, A.P.K., Herath, P.R.J & Mendis, K. (1991) Clustering of malaria infections within an endemic population: Risk of malaria associated with the type of housing construction. *American Journal of Tropical Medicine and Hygiene* **45**(1), 77-85.
- Garnham, P.C.C. (1988) Malaria parasite of man: life-cycles and morphology (excluding ultrastructure). In : *Malaria: Principal and Practice of Malariology*, volume 1. Wernsdorfer, W.H. & McGregor, I.A. (eds). Churchill Livingstone, London, pp61-96.
- Gillies M.T. (1988) Anopheline mosquitoes: vector behaviour and bionomics. In : *Malaria: Principal and Practice of Malariology*, volume 1. Wernsdorfer, W.H. & McGregor, I.A. (eds). Churchill Livingstone, London, pp453-486.
- Gibson, H.L, Trucker, J.W., Kaslow, D.C., Krettli, U.A., Collins, W.E., Kiefer, M.C., Bathurst, I.C., & Barr, P.J. (1992) Structure and expression of the gene for Pv200, a major blood-stage surface antigen of *Plasmodium vivax*. *Molecular and Biochemical Parasitology* **50**:235-334
- Greenwood B.M., Bradley, A.K., Greenwood, A.M., Byass, P., Jammeh, K., Marsh, K., Tulloch, S., Oldfield, F.S. & Hayes, R. (1987) Mortality and morbidity from malaria among children in rural area of The Gambia, West Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**: 478-486.
- Greenwood B.M. (1990) Letter in *Parasitology Today* **6**:180
- Hadley, T.J., Klotz, F.W. & Miller, L.H. (1986) Invasion of erythrocytes by malaria parasite: a cellular and molecular overview. *Annual Review of Microbiology* **40**:451-477
- Hartle, D. (1987) A primer of population genetics. Sinaur Associations, INC. Publishers, Sunderland, Massachusetts.

- Haynes J.D., Diggs C.L. Hines F.A. & Desjardins R.E. (1976) Culture of the human malaria parasite *Plasmodium falciparum*. *Nature* **263**:767-769
- Haridi, M.A. (1972) Parial exophily of *Anopheles gambiae* species B in the Khashm Elgirba area in eastern Sudan. *Bulletin of the Wold Health Organaization* **46**: 39-46
- Holder, A.A. & Freeman, R.R (1981) Immunization against blood-stage rodent malaria using purified parasite antigens. *Nature* **294**:361-364.
- Holder, A.A. & Freeman, R.R (1982). Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognised by immune serum and a monoclonal antibody. *Journal of Experimental Medicine* **156**: 1528-1538.
- Holder, A.A; Lockyer, M.J., Odink, K.G., Sandhu, J.S.; Riveros-Moreno, V.; Nicholls, S.C.; Hillman, Y.; Davey, L.S.; Tizard, M.L.V., Schwarz, R.T. & Freeman, R.R. (1985) Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. *Nature* **317**: 270-273.
- Holder, A.A, Sandhu, J.S., Hillman, Y., Davey, L.S., Nicholls, S.C., Cooper, H. & Lockyer, M.J. (1987) Processing of the precursor to the major merozoite surface antigens of *Plasmodium falciparum*. *Parasitology* **94**:199-208.
- Holder, A.A. (1988) The precursor to major merozoite surface antigens: structure and role in immunity. *Progress in Allergy*. **41**: 72-97
- Homeida, M., Ismail, A.A., Eltom, I., Mohamed, B. & Ali, H.M. (1988) Malaria and the Sudan floods. *Lancet* **11**, 92.
- Howells, R.E. & Davies, E.E. (1971) Nuclear division in the oocysts of *Plasmodium berghei*. *Annals of Tropical Medicine and Parasitology* **65**:451-459.
- Huber, W., Hurt, N., Mshinda, H., Jaquet, C., Koella, J.C. & Tanner, M. (1993) Sensitivity of *Plasmodium falciparum* field-isolates from Tanzania to chloroquine, mefloquine and pyrimethamine during in vitro cultivation. *Acta Tropica* **52**:313-316.
- Ibrahim, M. E., Awad-El-Kariem, F. M., Elhassan, I. M. & ElMubarak, E. R. M. (1991). A case of *Plasmodium falciparum* sensitive to chloroquine but resistant to pyrimethamine /sulphadoxine in Sennar, Sudan. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85**: 446.
- Ibrahim, A.M., Ali, F.R.A. and Ali M.E.M (1992) Assessment of chloroquine resistance of *P. falciparum* in children of Wad Medani (central Sudan). *Journal of Tropical Paediatric* **38**: 162-166.
- Isaacson, M. (1989) Airport malaria: a review. *Bulletin of the World Health Organization* **67**(6):737-743.
- Janse, C.J., Van der Klooster, P.F.J., Van der Kaay, H.J., Van der Ploeg, M. & Verdulve, J.P. (1986) DNA synthesis in *Plasmodium berghei* during asexual and sexual development. *Molecular and Biochemical Parasitology* **20**:173-183
- Janse, C.J. (1993) Chromosome size polymorphism and rearrangements in *Plasmodium*. *Parasitology Today* **9**(1): 19-22
- Jaureguiberry, G., Hatin, I., d'Auriol, L. & Galibert, G. (1990). PCR detection of *Plasmodium falciparum* by oligonucleotide probes. *Molecular and Cellular probes* **4**, 409-414.

- Jeffreys, A., Neumann, R. & Wilson, V. (1990) Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis, *Cell* **60**:473-485.
- Jongwutiwes, S., Tanabe, K., Nakazawa, S., Uemura, H. & Kanbara, H. (1991) Coexistence of GP195 alleles of *Plasmodium falciparum* in a small endemic area. *American Journal of Tropical Medicine and Hygiene* **44**:299-305.
- Jongwutiwes, S., Tanabe, K., Nakazawa, S., Yanagi, T., & Kanbara, H. (1992) Sequence variation in the tripeptide repeats and T cell epitopes in P190 (MSA-1) of *Plasmodium falciparum* from field isolates. *Molecular and Biochemical Parasitology* **51**: 81-90
- Joshi, H., Sumbarao, S.K., Rachavendra, K., & Sharama, V.P (1989) *Plasmodium vivax*: enzyme electrophoresis in isolates from Indian origin. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **83**: 179-81.
- Kemp, D.J., Thompson, J.K., Walliker, D. and Corcoran, L.M., (1987) Molecular karyotype of *Plasmodium falciparum*: Conserved linkage groups and expendable histidine-rich protein genes. *Proceedings of the National Academy of Sciences. USA.* **84**:7672-7676.
- Kemp, D., Cowman, A. and Walliker, D. (1990) Genetic diversity in *Plasmodium falciparum*. *Advances in Parasitology* **29**:75-149.
- Kerr, P., Ranford-Cartwright, L., & Walliker, D. Proof of intragenic recombination in *Plasmodium falciparum*. (in prep).
- Kilama, L.W. (1993) Malaria in Tanzania, past and present. 11th Annual Joint Scientific Conference. Tanzania, N.I.M.R., Arusha, February 1993.
- Kimura, M. & Crow, J.F (1964). The number of alleles that can be maintained in a finite population. *Genetics* **49**: 725-738.
- Kimura, E., Mattei, D., Di Santi, S.M. & Scherf, A. (1990). Genetic diversity in the major merozoite surface antigen of *Plasmodium falciparum*: high prevalence of a third polymorphic form detected in strains derived from malaria patients. *Gene* **91**: 57-62.
- Krajden S., Panisko, D.M., Tobe, B., Yang, J. and Keystone, J.S. (1991). Prolonged infection with *Plasmodium falciparum* in a semi-immune patient. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85**: 731-732
- Krogstad, D.J., Gluzman, I.Y., Kyle, D.E., Oduola, A.J., Martin, S.J., Milhouse, W.K. & Schlesinger, P.H. (1987) Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science* **238**:1283-1285
- Laskey, R.A. & Mills, A. (1975) Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *European Journal of Biochemistry* **56**:335-341
- Lienhard, C., Ghebray, R. & Condolfi, E. (1989) Does chloroquine resistance occur in refugee camps in Eastern Sudan? *Transactions of the Royal Society of Tropical Medicine and Hygiene* **83**: 486-487.
- Lindsay, S.W., Shenton, F.C., Snow, R.W. & Greenwood, B.M. (1989) Responses of *Anopheles gambiae* complex mosquitoes to the use of untreated bednets in The Gambia. *Medical and Veterinary Entomology* **3**:253-262

- Lines, J. and Armstrong, J.R.M. (1992). For a few parasites more: inoculum size, vector control and strain-specific immunity to malaria. *Parasitology Today* 8:381-383.
- Ling I.T. Ogun S.A. & Holder, A.A. (1994) Immunization against malaria with a recombinant protein. *Parasite Immunology* 16: 63-67
- Lockyer, M. J., Marsh, K., & Newbold, C. (1989) Wild isolates of *Plasmodium falciparum* show extensive polymorphism in T cell epitopes of the circumsporozoite protein. *Molecular and Biochemical Parasitology* 37: 275-280.
- Lyon J.A., Haynes J.D, Diggs C.L., Chulay J.D., Haiharis C.G. & Pratt-Rossiter J. (1987) Monoclonal antibody characterization of *Plasmodium falciparum* schizonts and merozoites: identification of additional processed products and a serotype-restricted repetitive epitope. *Journal of Immunology* 138:895-901
- Lyimo, E. (1993) The bionomics of the malaria mosquito *Anopheles gambiae sensu latu* in southeast Tanzania. PhD thesis, Wageningen Agricultural University.
- Marshall, V.M., Coppel, R.L., Martin, R.K., Oduola, A.M.J., Anders, R.F. & Kemp, D. (1991) A *Plasmodium falciparum* MSA-2 gene apparently generated by intragenic recombination between the two allelic families. *Molecular and Biochemical Parasitology* 45: 340-352.
- Marshall, V., Anthony, R.L., Bangs, M.J., Purnomo, Anders R.F. & Coppel, R.L. (1994) Allelic variation of the *Plasmodium falciparum* merozoite antigen2 (MSP-2) in geographically restricted area of Irian Jaya. *Molecular and Biochemical Parasitology* 63:13-21
- Maynard Smith, J., Smith, N.H., O'Rourke, M. & Spratt, B.G. (1993) How clonal are bacteria. *Proceedings of the National Academy of Sciences. USA.* 90: 4384-4388.
- McBride, J.S., Walliker, D., and Morgan, G. (1982) Antigenic diversity in the human malaria parasite *Plasmodium falciparum*. *Science* 217: 254-257.
- McBride, J.S., Newbold, C.I., & Anand, R., (1985) Polymorphism of a high molecular weight schizont antigen of the human malaria parasite *Plasmodium falciparum*. *Journal of Experimental Medicine* 161: 160-180.
- McBride, J.S & Heidrich, H.G (1987) Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites from an antigenic complex. *Molecular and Biochemical Parasitology* 23: 71-84
- McCutchan, T.F. Good, M.F. & Miller, L.H. (1989) Polymorphism in the circumsporozoite (CS) protein of *Plasmodium falciparum*. *Parasitology Today* 5(5):143-146.
- Miller, L.H., Robert, T., Shahabuddin, M. & McCutchan, T.F. (1993) Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). *Molecular and Biochemical Parasitology* 59: 1-14
- Molineaux, L. & Gramiccia, G. (1980) The Garki Project: research on epidemiology and control of malaria in Sudan savanna of west Africa. *World Health Organization*, Geneva.
- Molineaux, L., Muir, D.A., Spencer, H.C. & Wernsdorfer, W.H. (1988) The epidemiology of malaria and its measurements. In : *Malaria: Principal and Practice of Malariology*, volume 1. Wernsdorfer, W.H. & McGregor, I.A. (eds). Churchill Livingstone, London, pp999-1089.
- Omer, A. H. S.(1978) Response of *Plasmodium falciparum* to oral chloroquine. *American Journal of Tropical Medicine and Hygiene* 27: 853-857.

Omer, S.M. and Cloudesly-Thompson, J.L. (1970) Survival of *Anopheles gambiae* Giles through a 9-month dry season in Sudan. *Bulletin of the World Health Organization* 42:9319-330.

Patarroyo, M.E., Amador, R., Clavijo, P., Moreno, A., Guzman, F., Romero, P., Taxon, R., Franco, A., Murillo, L.A., Ponton, G. & Trujillo, G. (1988) A synthetic vaccine protects humans against challenge with asexual blood stage of *Plasmodium falciparum*. *Nature* 332: 158-160.

Pace, T., Ponzi, M., Dore, E., Janse, C., Mons, B. & Frontali, C. (1990) Long insertion within telomeric contribute to chromosome size polymorphism in *Plasmodium bergeri*. *Molecular and Cellular Biology*. 10(12): 6759-6764.

Padua, R.A. (1981) *Plasmodium chabaudi*: Genetics of resistance to cloroquine. *Experimental Parasitology*, 52: 419-426.

Payne, D. (1987) Spread of chloroquine resistance in *Plasmodium falciparum*. *Parasitology Today* 3: 241-246.

Perrin, L.H., Merkli, B., Loche, M., Chizzolini, C., Smart, J. & Richle R. (1984) Antimalarial immunity in *Saimiri* monkey: immunisation with surface components of asexual blood stages. *Journal of Experimental Medicine* 160: 441-451.

Peterson, D.S., Walliker, D. & Wellems, T.E. (1988a) Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proceedings of the National Academy of Sciences. USA*. 85: 9114-9118.

Peters, W. (1987) Resistance in human malaria 1: 4-aminoquinolines and multiple resistance. In: Chemotherapy and resistance in malaria, Vol. 2. Peters W. (editor). Academic Press, London. pp659-786.

Peterson, M.G., Coppel, R.L., Moloney, M.B. & Kemp, D.J., (1988b) Third form of the precursor to the major merozoite surface antigens of *Plasmodium falciparum*. *Molecular Cellular Biology* 8: 2664-2667.

Peterson, D., Milhous, W.L. & Wellems, T.E. (1990) Molecular basis of different resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. *Proceedings of the National Academy of Sciences. USA*. 87: 3018-3022.

Pinswasdi, C., Thaithong, S., Beale, G.H., Fenton, B., Webster, H.K. & Pavanand, K. (1987) Polymorphism of proteins in malaria parasites following mefloquine treatment. *Molecular and Biochemical Parasitology* 23:159-164.

Ponnudurai, T., Lensen, A.H.W., van Gemert, G.J.A., Bolmer, M.G & Meuwissen, J.H.E.Th. (1991) Feeding behaviour and sporozoite ejection by infected *Anopheles stephensi*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 85: 175-180.

Prensier, G. and Slomianny, C. (1986) The karyotype of *Plasmodium falciparum* determined by ultrastructural serial sectioning and 3D reconstruction. *The Journal of Parasitology* 72:731-736.

Ranford-Cartwright, L., Balf, P., Carter, R. & Walliker, D. (1991). Genetic hybrids of *Plasmodium falciparum* identified by amplification of genomic DNA from single oocysts. *Molecular and Biochemical Parasitology* 49:239-44.

Ranford-Cartwright, L., Balf, P., Carter, R. & Walliker, D. (1993). Frequency of cross-fertilization in the human malaria parasite *Plasmodium falciparum*. *Parasitology* 107:11-18.

- Read, A.F., Narara, A., Nee, S., Keyemer, A.E. & Day, K (1993). Gametocyte sex ratio as indirect measures of outcrossing rates in malaria. *Parasitology* **104**: 387-395.
- Rosario, V. (1976) Genetics of chloroquine resistance in malaria parasites. *Nature* **261**:585-586.
- Rosario, V. (1981) Cloning of naturally occurring mixed infections of malaria parasites. *Science* **212**: 1037-1038.
- Rosenberg, R. & Rungsiwongse, J. (1991) The number of sporozoites produced by an individual malaria oocyst. *American Journal of Tropical Medicine & Hygiene* **45**: 574-577.
- Rosenberg, R., Rungsiwongse, J., Kangsadalmpai, S. Sattabongkot, J.; Suwanabun, N.; Chaiyaroj, S. & Mongkolsuk, S. (1992) Random mating of natural *Plasmodium* populations demonstrated from individual oocysts. *Molecular and Biochemical Parasitology* **52**:129-34
- Saiki, R., Scharf, S., Faloona, F., Mullis, K., Horn, G., Erlich, H. & Arnheim, N. (1985). Enzymatic amplification of a-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350-1354.
- Saeed, B.O, Hassabalrasoul, M.A., Ibrahim, K. E., Abdel-Karim, E. I., Salih, S.A., Hassan, I. M., Khider, S. & Bayoumi, R.A.(1990) Plasma chloroquine measurement in the evaluation of *Plasmodium falciparum* sensitivity. *Journal of Tropical Medicine and Hygiene* **93**, 393-396.
- Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press.
- Sanderson, A., Walliker, D. & Molez, J-F. (1981) Enzyme typing of *Plasmodium falciparum* from some African and other old world countries. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **75**:263-267.
- Saul, A., Lord, R., Jones, G., Geysen, H.M., Gale, J. & Mollard, R. (1989), Cross-reactivity of antibody against an epitope of the *Plasmodium falciparum* second merozoite surface antigen. *Parasite Immunology* **11**:593-601.
- Scherf A., Mattei D. & Sarthou J.-L. (1991). Multiple infections and unusual distribution of block 2 of the MSA1 gene of *Plasmodium falciparum* detected in West African clinical isolates by the polymerase chain reaction analysis. *Molecular and Biochemical Parasitology* **44**:297-300.
- Sharkey, A., Langsley, G., Patarapotikul, J., Mercereau-Puijalon, O., McLean, A. & Walliker, D. (1988) Chromosome size variation in the malaria parasite of rodents *Plasmodium chabaudi*. *Molecular and Biochemical Parasitology* **28**:47-54.
- Siddiqui, W.A, Tam, L.Q., Kramer, K.J., Hui, G.S.N., Case, S.E., Yamaga, K.M., Chang, S.P., Chan, E.B.T. & Khan, S. C. (1987) Merozoite surface coat precursor protein completely protects Aotus monkeys against *Plasmodium falciparum* malaria. *Proceedings of the National Academy of Sciences. USA.* **84**: 3014-3018.
- Simmons, D. Woollett, G., Bergin-Cartwright, M., Kay, D. & Scaife, J.G (1987). A malaria protein exported into a new compartment within the host erythrocyte. *EMBO Journal* **6**: 485-491.
- Sinden, R.E., Canning, E.U. & Spain, B.J. (1976) Gametogenesis and fertilisation in *Plasmodium yoelii nigeriensis*; a transmission electron microscope study. *Proceedings of the Royal Society (London) B.* **193**:55-76
- Sinden, R.E. (1978) Cell Biology. In: Rodent malaria pp85-168. Killick-Kendrick, R., and Peters, W. eds. Academic Press, London.

- Sinden, R.E. & Hartley, R.H. (1985) Identification of the meiotic division of malaria parasites. *Journal of Protozoology* **32**:742-744.
- Sinnis, P. and Wellems, T.E. (1988) Long-range restriction maps of *Plasmodium falciparum* chromosomes: Crossing-over and size variation among geographically distinct isolates, *Genomics* **3**:287-295.
- Smalley, M.E. & Sinden, R.E (1977) *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* **74**:1-8
- Smythe, J.A., Coppel, R.L., Brown, G.V., Ramasamy, R., Kemp, D.J. & Anders, R.F. (1988). Identification of two integral membrane protein of *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences. USA.* **85**: 5195-5199.
- Smythe, J.A., Peterson, M.G., Coppel, R.L., Saul, A.J., Kemp, D.J. and Anders, R.F. (1990) Structural diversity in the 45-kilodalton merozoite surface antigen of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **39**: 227-234
- Smythe, J.A., Coppel, R.L., Day, K.P., Martin, R.K., Oduola, A.M.J. , Kemp, D.J. & Anders, R.F.(1991) Structural diversity of the *Plasmodium falciparum* merozoite surface antigen MSA-2. *Proceedings of the National Academy of Sciences. USA.* **88**: 1751-1755.
- Smith, T., Charlwood, J.D, Kihonda, J., Mwankusye, S., Billingsley, P., Meuwissen, J., Lyimo, E., Takken, W., Teuscher, T. & Tanner, M. (1993) Absence of seasonal variation on malaria parasitaemia in area of intense seasonal transmission. *Acta Tropica* **54**: 55-72.
- Snewin, V.A.; Herrera, M. Sanchez, G.; Scherf, A.; Langsley, G. & Herrera, S. (1991) Polymorphism of alleles of the merozoite surface antigens MSA-1 and MSA-2 in *Plasmodium falciparum* wild isolates from Colombia. *Molecular and Biochemical Parasitology* **49**:265-276.
- Snow, R.W., Armstrong Schellenberg, J.R.M., Peshu, N., Foster, D., Newton, C.R.J.C., Winstanley, P.A., Mwangi, I., Waruiru, C., Wain, P.A., Newbold, C. & Marsh, K. (1993) Periodicity and space-time clustering of severe childhood malaria on the coast of Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **87**:386-390.
- Spencer, H.C.(1985) Drug resistance malaria-changing pattern mean difficult decisions. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**: 748-758.
- Stanley, H.A., Howard, R.F., & Reese, R.T. (1985) Recognition of a Mr 56K glycoprotein on the surface of *Plasmodium falciparum* merozoite by mouse monoclonal antibodies. *Journal of immunology* **134**:3439-3444.
- Sudan (1986) *Annual statistical report*. Khartoum: Ministry of Health.
- Tait, A. (1981) Analysis of protein variation in *Plasmodium falciparum* by two dimensional gel electrophoresis. *Molecular and Biochemical Parasitology* **2**: 205-218.
- Tanabe, K., Mackay, M., Goman, M. and Scaife, J.G. (1987). Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *Journal of Molecular Biology* **195**:273-287
- Tanner, M., De Savigny, D., Mayobana, C., Hatz, C., Burnier, E., Tayari, S. & Degremont, A. (1991) Morbidity and mortality at Kilombero, Tanzania, 1982-88. In: Disease and mortality in sub-saharan Africa (eds) Freachem, R.G. & Jamison, D.T. Oxford University Press.

- Taylor, R.R., Smith, D., Robinson, J.V., Riley, E.M. & McBride, J. (1993) Human immune recognition of the *Plasmodium falciparum* merozoite surface protein MSP-2. 5th malaria meeting, British Society for Parasitology, 22nd-24th September.
- Teusher, T., Armstrong Schellenberg, J.R., Bastos de Azevedo, I., Hurt, N., Smith T., Hayes, R., Masanja, H., Sliva, Y., Lopez, M.C., Kitua, A., Kilama, W., Tanner, M. & Alonso, P.L. (1994) SPf66, a chemically synthesised subunit malaria vaccine, is safe and immunogenic in Tanzanians exposed to intense malaria transmission. *Vaccine* 12(4): 328-336.
- Thaithong, S., Sueblinwong, T. and Beale, G.H. (1981) Enzyme typing of some isolates of *Plasmodium falciparum* from Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 75:268-270.
- Thaithong, S., Beale, G.H. and Chutmongkonkul, M. (1983) Susceptibility of *Plasmodium falciparum* to five drugs: an in vitro study of isolates mainly from Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 77:228-231.
- Thaithong, S., Beale, G.H., Fenton, B., McBride, J., Rosario, V., Walker, A. & Walliker, D. (1984) Clonal diversity in a single isolate of the malaria parasite *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 78:242-245.
- Thaithong, S., Siripoon, N., Seugorn, N., Bunnag, D. and Beale, G.H. (1989) Electrophoretic variations of enzymes in isolates of *Plasmodium falciparum*, *P. malariae* and *P. vivax* from Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 83:602-605.
- Thomas, A.W., Carr, D.A., Carter, J.M., & Lyon, J.A. (1990) Sequence comparison of allelic forms of *Plasmodium falciparum* merozoite surface antigen MSA2. *Molecular and Biochemical Parasitology* 43: 211-220.
- Tibayrenc, M., Kjellberg, F. & Ayala, F.J. (1990) A clonal theory of parasitic protozoa: The population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas* and *Trypanosoma* and their medical and taxonomical consequences. *Proceedings of the National Academy of Sciences. USA.* 87: 2414-2418.
- Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Breniere, S.F., Darde, M-L., & Ayala, F.J. (1991) Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. *Proceedings of the National Academy of Sciences. USA.* 88. 5129-5133.
- Tolle, R., Fruh, K., Doumbo, O., Koita, O., N'Diaye, M., Fischer, A., Dietz, K. & Bujard, H. (1993) A prospective study of the association between the human humoral immune response to *Plasmodium falciparum* blood stage antigen gp190 and control of malaria infections. *Infection and immunity* 61(1):40-47
- Trager, W. and Jensen, J.B. (1976). Human malaria parasites in continuous culture. *Science* 193: 673-675.
- Trenholme, K. & Philips, R. S. (1989) The use of murine feeder cells in the cultivation of *Plasmodium falciparum* asexual blood stages. *Parasitology Research* 75: 518-521.
- Triglia, T., Wellems, T.E. & Kemp, D.J. (1992). Towards a high resolution map of the *Plasmodium falciparum* genome. *Parasitology Today* 8: 225-229.
- Van Der Ploeg, L.H.T., Smith, M., Ponnudurai, TH. Vermeulen, A., Meuwissen, J.H.E.T. & Langsley, G. (1985) Chromosome-sized DNA molecule of *Plasmodium falciparum*. *Science* 229:658-661.

- Walker-Jonah, A., Dolan, S.A., Gwadz, R.W., Panton, L.J. & Wellems, T.E. (1992). An RFLP map of the *Plasmodium falciparum* genome, recombination rate and favoured linkage groups in a genetic cross. *Molecular and Biochemical Parasitology* **51**:313-320.
- Walliker, D., Carter, R. & Morgan, S. (1973) Genetic recombination in *Plasmodium berghei*. *Parasitology* **66**:309-320.
- Walliker D., Carter, R., Sanderson, A. (1975) Genetic studies of *Plasmodium chabaudi*: recombination between enzyme markers. *Parasitology* **70**:19-24
- Walliker, D. (1985) Characterisation of *Plasmodium falciparum* of different countries. *Annals de la Science Belge de Medicine Tropicale* **65**: (suppl.2) 69-77.
- Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F., Szarfman, A., London, W.T., Corcoran, L.M., Burkot, T.R. and Carter, R. (1987). Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science* **236**, 1661-1666
- Walliker, D. (1989) Implications of genetic exchange in the study of protozoan infections. *Parasitology* **99**: (suppl.) s49-s58.
- Walliker, D. (1991) Malaria parasite: randomly interbreeding or 'clonal' populations? *Parasitology Today* **7**(9): 232-235.
- Weber, J.L., Leininger, W.M. & Lyon, J.A. (1986) Variation in gene encoding a major merozoite surface antigen of the human malaria parasite *Plasmodium falciparum*. *Nucleic Acids Research* **14**:3311-3322.
- Weir, S.B. (1992) Independence of VNTR alleles defined as fixed bins. *Genetics* **130**:873-887.
- Wellems, T.E., Panton, L.J., Gluzman, I.Y., Rozario, V., Gwadz, R.W., Walker-Jonah, A. & Krogstad, D.J. (1990) chloroquine resistance not linked to *mdr*-like genes in a *Plasmodium falciparum* cross. *Nature* **345**:253-255
- Wellems, T.E., Walker-Jonah, A. & Panton, L.J., (1991) Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. *Proceedings of the National Academy of Sciences. USA.* **88**:3382-3386.
- Wernsdorfer, G. & Wernsdorfer, W. (1967) Malaria in the middle Nile basin & its bordering regions. *Zeit. Trop. Med. Parasitol.* **18**:17-44
- Wernsdorfer, W. H. (1991) The development and spread of drug resistant malaria. *Parasitology today* **7**(11), 297-303.
- Whitfield, D., Curtis, C.F., White, G.B., Targett, G.A., Warhust, D.C. & Bradley, D.J. (1984) Two cases of falciparum malaria acquired in Britain. *British Medical Journal* **289**:1607-1609.
- Wilson, C.M., Serrano, A.E., Wasley, A., Bogenschutz, M.P., Shankar, A.H. & Wirth, D.F. (1989) Amplification of a gene related to mammalian *mdr* genes in drug resistant *Plasmodium falciparum*. *Science* **224**:1184-1186.
- World Health Organization (1978) Primary Health care. Report on international conference on primary health care, Alma-Ata, USSR, 6-12 September 1978. Publication of World Health Organization, Geneva.

World Health Organization (1980) Malaria control in countries where time-limited eradication is impracticable at present. Technical report series No 537. Geneva.

World Health Organization (1985) Bench aids for diagnosis of malaria. set no. 1. Plates No 1-4.

Wunderlich, F., Falk, H., & Konigk, E. (1980) Nucleosomal organization of chromatin in *Plasmodium knowlesi*. *Journal of Parasitology* 66:1063-1065.

Zolg, J.W., Chen, G.-X. & Plitt, J.R. (1990) Detection of pyrimethamine resistance in *Plasmodium falciparum* by mutation-specific polymerase chain reaction. *Molecular and Biochemical Parasitology* 39: 257-267

APPENDIX 1. Summary of the model used for population genetic analysis.

The model used in the analysis of the oocyst data in chapter 6 calculates the expected proportions of the possible oocyst genotypes assuming linkage equilibrium and sub structuring of the *Plasmodium* population between people. The model comprises an infinite population of infected individuals from which oocysts are formed by random union of gametes within mosquitoes. This assumption was based on the fact that in an experimental laboratory cross different gametes of *P. falciparum* were found to mate at random in the mosquito vector (Ranford-Cartwright *et al.*, 1993). It also assumed that MSP-1 and MSP-2 are in linkage equilibrium, (Triglia *et al.*, 1992; Conway & McBride, 1991) and thus the frequency of each of the six possible *Plasmodium* clones in the population is the product of allele frequencies at the two loci.

Sub structuring of the Plasmodium population

1. number of clones

Infected people in the village have varying numbers of clones, from 1 to 6. We describe the population as having some distribution of numbers of infections, and thus some distribution of numbers of clones. (Some multiple infections will be of the same clone.) We have modelled this distribution as being negative binomial in form. This is a discrete distribution with the variance always greater than the mean.

$$f_y(n) = \binom{n+r-1}{n} p^r q^n = \frac{G(n+r)}{G(n+1)G(r)} \quad n=0,1,\dots_\infty$$

Since all oocysts must come from infected individuals the zero class of infections is removed. The distribution is truncated to between 1 and 8 infections. (increasing the possible numbers of infections did not alter the shape of the distribution significantly for the range of parameter values used.) The shape of the distribution is determined by varying the mean, m , and

variance, v , where $m = \frac{rq}{p}$ and $v = \frac{rq}{p^2}$

We have summarised the distribution by the proportion of people with single clones, and the mean number of clones per person.

2. Combinations of clones

Given the numbers of clones present in an individual the frequency of particular combinations of clones is given by the multinomial sampling theorem.

$$f_{x_1, x_2 \dots x_k} (x_1 \dots x_k) = \frac{n!}{\prod_{i=1}^k n_i!} \prod_{i=1}^k p_i^{n_i} \quad \sum_{i=1}^k x_i = n, i = 1 \dots k, x_i = 0 \dots n$$

From the above the expected proportion of people with particular numbers and combinations of clones is calculated.

3. Sampling a single oocyst

A further sampling process is needed before the expected proportions of oocyst genotypes can be calculated. Since only a single oocyst per mosquito is scored this will not always accurately represent the clones present in the blood of the individual human host. When there is more than one clone present we assume that there is random union of gametes within the mosquito, which will still give rise to some homozygous oocysts for example. Given the small sample of oocysts this could bias the estimated *Plasmodium* population structure or estimated allele frequencies. The binomial coefficient is used to determine the expected proportions of diploid gametes given the combinations of clones present in blood.

4. Maximum Likelihood estimation of model parameters.

The expected proportions of each oocyst genotype class depend on the allele frequencies at the two loci, (3 parameters), and m and v , the parameters describing the negative binomial distribution. By iterating across a range for each parameter expected oocyst genotype frequencies are generated and these alternative models compared with the observed data by the method of maximum likelihood.

$$LnL = \sum_{i=1}^{ngenotypes} O_i Ln p_i$$

O = observed number of genotype class i p_i = expected proportion in genotype class i

The support range for each parameter is given by the range for which the LnL is within 2 of the maximum. This can be thought of as analogous to a 95% confidence interval.

Appendix 2. Publications from this work.

Babiker, H.A., Creasey, A.M., Fenton, B., Bayoumi, R.A., Arnot, D.E & Walliker, D. (1991). Genetic diversity of the human malaria parasite *Plasmodium falciparum* in a village in Eastern Sudan. I . Diversity in enzymes, 2D-PAGE proteins and antigens. *Trans. Roy. Soc. Trop. Med. Hyg.* **85**, 572-577.

Babiker, H.A., Creasey, A.M., Bayoumi, R.A., Walliker, D. & Arnot, D.E. (1991). Genetic diversity of the human malaria parasite *Plasmodium falciparum* in a village in Eastern Sudan. II. Drug resistance, molecular karyotypes and the *mdr-1* genotype in recent field isolates. *Trans. Roy. Soc. Trop. Med. Hyg.* **85**, 578-583.

Foley, M. Ranford-Cartwright, L. C. and Babiker, H.A. (1992) Rapid and simple method for isolating malaria DNA from fingerprick samples of blood. *Mol. Biochem. Parasitol.*, **53**, 241-244.

Bayoumi, R.A., Creasey, A.M., Babiker, H.A., Carlton, J., Sultan, A., Satti, G., Sohal, A., Walliker, D. & Arnot, D.E. (1993) Drug response and genetic characterisation of *P.falciparum* clones recently isolated from a Sudanese village. *Trans. Roy. Soc. Trop. Med. Hyg.* **87**: 454-458.

Walliker, D., Ranford-Cartwright, L. C., Babiker, H. A (1993) Genetic recombination in field populations of *Plasmodium falciparum*. In "Genome analysis of protozoan parasites" (ed) Morzaria, S. P. Proceedings of a workshop held at Ilrad Nairobi, Kenya, 11-13th November 1992.

Babiker H. A. and Ranford-cartwright, L. C. (1993) Diversity of two antigen genes of malaria parasite detected by PCR and ECL3'-oligolabelling system. *ECL Highlights*. S550/6505/93/1

Babiker, H. A., Ranford-Cartwright, L. C., Sultan, A., Satti, G. and Walliker, D. (1994) Genetic evidence for the occurrence of R1 chloroquine resistant *P.falciparum* parasites in patients with relapses of clinical malaria, in a village in Sudan. *Trans. Roy. Soc. Trop. Med. Hyg.* **88**:

Babiker, H. A., Ranford-Cartwright, L. C., Currie, D., Charlwood, D., Billingsley, P., Teuscher, T., Walliker, D. Random-mating in natural populations of malaria parasite *Plasmodium falciparum*. *Parasitology* (in press).

Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens

Hamza Ali Babiker^{1,2}, Alison M. Creasey¹, Brian Fenton¹, Riad A. L. Bayoumi³, David E. Arnot¹ and David Walliker¹
¹Institute of Cell, Animal and Population Biology, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JN, Scotland, UK; ²Malaria Administration, Ministry of Health, Khartoum, Sudan; ³Department of Biochemistry, Faculty of Medicine, University of Khartoum, Sudan

Abstract

Twenty-nine *Plasmodium falciparum* isolates from patients in Asar village, eastern Sudan, were characterized for variation in 18 different genetically controlled characters, including iso-enzymes, proteins detected by two-dimensional polyacrylamide gel electrophoresis and blood-stage antigens. Considerable allelic diversity in the genes determining these characters was detected. Each isolate contained genetically distinct parasites. Fifteen individuals were infected with more than one parasite genotype. The diversity of parasite types is most probably generated by recombination during mosquito transmission of mixed parasite clones.

Introduction

Malaria is one of the most serious health problems in Sudan, accounting for some 40% of all infectious diseases (EL GADDAL, 1986; SUDAN, 1986). The predominant parasite species is *Plasmodium falciparum*, which is responsible for about 90% of reported malaria cases (OMER, 1978). Until now, however, no information has been available on the genetic composition of *P. falciparum* populations in Sudan, either in regions of endemic malaria or during seasonal malaria epidemics. Such information is needed for the planning of new control measures, especially in view of the increasing number of drug-resistant forms of this parasite being reported (BAYOUMI *et al.*, 1989).

Numerous characters of *P. falciparum* exhibit diversity, including drug response (PETERS, 1987), antigens (MCBRIDE *et al.*, 1982), iso-enzymes (SANDERSON *et al.*, 1981), proteins detected by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (FENTON *et al.*, 1985), as well as deoxyribonucleic acid sequence and chromosome polymorphisms (KEMP *et al.*, 1990). Previous studies making use of such variable markers have established that considerable diversity exists among the parasites of a single country, that infections with more than one parasite clone are common, and that geographical variations occur in the frequency of variant forms of certain characters (CREASEY *et al.*, 1990). A study of *P. falciparum* infections in Papua New Guinea demonstrated that different villages in the same area showed variations in the prevalence of two S-antigen serotypes (FORSYTH *et al.*, 1989).

In this and the following paper (BABIKER *et al.*, 1991) we report a detailed study of the genetic structure of *P. falciparum* in a single village in eastern Sudan. We examine variation in 18 genetically controlled characters among *P. falciparum* isolates collected in the village during a two-week period in

the seasonal epidemic of 1989. The characters examined in this paper are iso-enzymes, 2D-PAGE proteins and antigens. The variant forms of these markers are due to allelic variation of each respective gene, as shown by crossing experiments (WALLIKER *et al.*, 1987; FENTON & WALLIKER, 1990). In the accompanying paper, the drug response, molecular karyotypes and *mdr* genotypes of the same parasites are discussed. We show that considerable diversity in all these characters exists in this parasite population, all patients examined harbouring different genotypes.

Materials and Methods

Study area

Asar village is approximately 18 km south-east of Gedaref, one of the principal towns of the eastern region of Sudan (Fig. 1), and has about 4000 inhabitants. The villagers practice traditional subsistence agriculture centred around sorghum (*dura*) cultivation, which is dependent upon seasonal rainfall (July–October). Malaria transmission is also seasonal, reaching its peak just after the rainy season, from around September to November.



Fig. 1. Map of Sudan showing area of study in eastern Sudan.

Isolates of *P. falciparum*

Twenty-nine isolates were collected (with informed consent) from patients attending a small clinic in the village who, on examination, were found to have high *P. falciparum* parasitaemias. The patients included sibling pairs from 2 houses, providing isolates 117/89 and 122/89 (brothers) and 106/89 and 110/89 (brother and sister). Patients with positive blood slides were treated with chloroquine immediately after sample collection. Collections were made between 24 October and 4 November 1989.

The isolates were deep-frozen in ampoules in liquid nitrogen for subsequent transport to Edinburgh. After thawing, the isolates were cultured by a modification of the method of TRAGER & JENSEN (1976), which involved establishing cultures in flasks gassed with a mixture of 1% oxygen, 3% carbon

dioxide and 96% nitrogen. Mouse peritoneal wash cells were added to enhance parasite growth (TRENHOLME & PHILLIPS, 1989).

Enzyme electrophoresis

Variant forms of 3 enzymes, glucose phosphate isomerase (GPI), adenosine deaminase (ADA), and peptidase (PEP), were identified by cellulose acetate electrophoresis of extracts of parasites freed from host red cells by saponin lysis (SANDERSON *et al.*, 1981).

Two-dimensional polyacrylamide gel protein electrophoresis

Parasites in culture were incubated with ^{35}S methionine and subjected to 2D-PAGE, as described by FENTON *et al.* (1985). Using this technique, 15 proteins have been identified which exhibit allelic

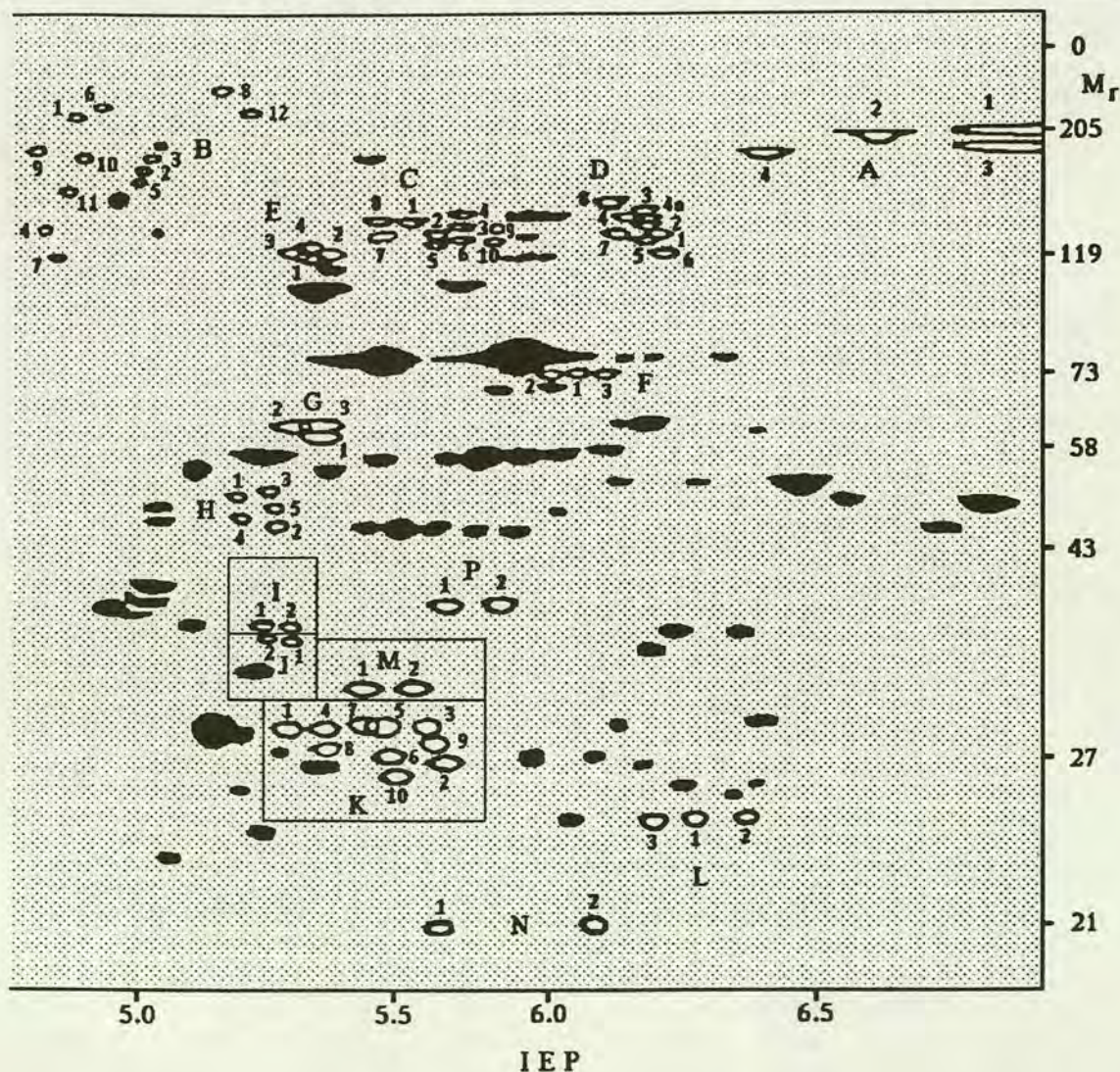


Fig. 2. Two-dimensional polyacrylamide gel electrophoresis patterns of proteins of *Plasmodium falciparum*. Composite diagram showing variant proteins (letters) and their allelic variants (numbers). IEP=isoelectric point; M_r =relative molecular mass. Solid spots indicate proteins having the same charge and size in different parasite clones, open spots indicate proteins varying in charge and/or size in different clones.

variation in different parasite clones, the variation being in either or both isoelectric point or molecular weight. Nine of these proteins, denoted C, D, F, G, I, K, L, N and P, were used to characterize the parasites in this study. The variant forms of each protein are designated by a number according to the classification described by FENTON (1987); these proteins, as well as new forms detected in the present work, are illustrated in Fig. 2.

Antigens

Variant forms of 3 asexual stage antigens, MSA-1, MSA-2 and Exp-1, were identified by the indirect immunofluorescence assay (IFA) described by McBRIDE *et al.* (1982), using monoclonal antibodies (Mabs) recognizing variant epitopes on each molecule.

MSA-1 and MSA-2 are merozoite surface antigens, different allelic forms of which exhibit serological diversity detectable by Mabs (McBRIDE *et al.*, 1985; FENTON *et al.*, 1989). 35 allelic variants of MSA-1, and 8 of MSA-2, have now been identified using these Mabs, the alleles of each antigen being identified by numbers according to their Mab reactivities (CONWAY & McBRIDE, 1991); this system of numbering is used in the present work. Isolates containing mixtures of parasites with different alleles of these antigens were detected by a double-labelling technique. This in-

cluded mixing Mabs of different isotypes together on the same parasite preparation, and staining with a mixture of isotype-specific fluorescein-labelled and rhodamine-labelled antibodies (CARTER *et al.*, 1989).

The Exp-1 antigen is located in the parasitophorous vacuole and in membranous structures in the infected cell (KEMP *et al.*, 1986). Two allelic forms have been detected, differentiated by Mab 5-1, which differ by a single amino acid at position 136 (SIMMONS *et al.*, 1987).

Results

Considerable diversity was found among the parasites of the 29 isolates studies. Table 1 provides details of the characteristics of the parasites found in each isolate.

At least 2 variant (allelic) forms of each character examined were present among the parasites from Asar. For example, the enzyme GPI was present as two forms, GPI-1 and GPI-2. The most variable characters were MSA-1, which possessed 8 alleles, and the 2D-PAGE proteins D and K, which possessed 7 alleles each. Each isolate possessed a different combination of the alleles of each marker. In 15 isolates, more than one form of at least one character was detectable. Since *P. falciparum* is haploid in the blood stages, these isolates must contain mixtures of more than one parasite genotype. The precise geno-

Table 1. Genetic characteristics of *Plasmodium falciparum* isolates from Asar village, eastern Sudan

Isolate	2D-PAGE proteins ^a									Enzymes ^b			Antigens ^c		
	C	D	F	G	I	K	L	N	P	GPI	PEP	ADA	MSA-1	MSA-2	Exp-1
101/89	2	4a+5	2	1	1	6+1	3+1	—	1	1	1	1	28+20	2	+/-
102/89	—	—	1	1	1	10	3	2	1	1	1	1	49	2	—
104/89	2	3	1	1	1	6	1	2	1	1	1	1	28+20	2+7	-/+
105/89	2	8	1	2	1	3	3	—	2	1	1	2	20	4	+
106/89	9	5	1	1+2	1	3+1	3	1	1	1	1	1	20+26?	2+8	+
107/89	3	2	2	1	1	—	1	1+2	1	1	1	1	20	2	—
108/89	2	3	1	1	1	3	3	—	1	1	1	1	49+26?	2	—
109/89	—	—	1+2	2	—	—	1	2	1	2	3	1	15	2	—
110/89	—	4a+5	2	1	1	9+10	3	2	1	1	1	1	20	2	—
111/89	10	3	2	1	1	3	1	—	1	2	1	1	50	2	-/+
112/89	10	5	2	1	1	1	3	—	1	1	1	1	28	2	—
113/89	—	9	1	1	1	3	3	1	1	1	1	1	20+28	2+7	+
114/89	—	6	1	1	2?	—	3	—	2	1	1	44	2	+	—
115/89	—	4a	2	1	1	—	1	2	1	2	1	1	32	2	—
116/89	—	3	2	1	1	3	1	2	1	1	2	1	20	2	—
117/89	—	3	1	1	1	6	3	2	1	1	1	1	50	2	—
118/89	3	—	2	1	1	—	1	1+2	1	1	1	1	28	2	—
119/89	—	3	1	1	1	—	2	2	1	1	1	1	20	4	—
120/89	1?	3	1	1	1	3	3	2	1	1	1	1	49	2	—
121/89	9	5+3	1	1	1	—	3	—	1	1	1	1	20+28	2+8	+
122/89	—	3	2	1+2	1	10	1	2	1	1	1	1	28	2	—
123/89	10	4a+5	2	1	1	6	1+3	—	1	1	1	1	28	2	+
124/89	3	2	2	1	1	3	3	1	1	1	1	1	49+20	2+7	+
125/89	6	4a+8	2	1	1	3	3	—	1	1	1	1	28+20	2+7	+
126/89	10	4a	1	1	1	11?	1+3	—	1	1	1	1	28+20	4	—
127/89	—	—	2	1	1	—	1	1	1	1	1	1	20	4	+
128/89	—	—	1	1	1	2+10	3+1	1+2	1	1	1	1	50	2	+/-
129/89	—	3	2	1	1	10	3	—	1	1	1	1	50	2	—
130/89	—	—	1+2	1	1	3	3	1	1	1	1	1	49+20	2	+

^a2D-PAGE proteins are polymorphic parasite proteins detected by two-dimensional polyacrylamide gel electrophoresis; letters refer to proteins determined by different genetic loci, and numbers indicate allelic forms of each protein; — indicates that a protein could not be detected, ? indicates uncertain identity of the allele.

^bGPI, ADA and PEP are variant forms of enzymes detected by electrophoresis (full names are given in the text).

^cMSA-1 and MSA-2 are merozoite surface antigens; numbers indicate allelic forms detected by monoclonal antibodies; ? indicates uncertain identity of the allele. Exp-1 is a blood-stage antigen; +/- or -/+ denote a mixed infection, the first symbol indicating the predominant type.

Table 2. Characteristics of *P. falciparum* isolates of two household pairs in Asar

	C	D	F	2D-PAGE proteins ^a			L	N	P	Antigens ^a		
				G	I	K				MSA-1	MSA-2	Exp-1
Pair 1												
106/89	9	5	1	1+2	1	3+1	3	1	1	20+26(?)	2+8	+
110/89	—	4a+5	2	1	1	9+10	3	2	1	20	2	—
Pair 2												
117/89	—	3	1	1	1	6	3	2	1	50	2	—
122/89	—	3	2	1+2	1	10	1	2	1	28	2	—

^aSee Table 1 for explanation.

type present in each isolate could be identified by cloning, although this was not done in the present work. Such multiclonal infections have been reported in earlier studies on *P. falciparum* in other countries (CREASEY *et al.*, 1990).

With regard to the sibling pairs, neither member of each pair was infected with identical genotypes (Table 2). Both members of pair 1 showed a mixed infection with not less than 2 different clones. In pair 2, each infection appeared to be of clonal type, but each was a different genotype. The parasites from each member of the 2 pairs could be distinguished by at least 5 different characters.

The following observations were made concerning the frequencies of the different markers.

(i) *Enzymes*. Two forms of the enzymes GPI and ADA, and 3 of PEP, were detected in Asar. GPI-1, ADA-1 and PEP-1 were the most common forms. ADA-2, PEP-2 and PEP-3 were each found in only single isolates.

(ii) *2D-PAGE proteins*. The variation seen in the 2D-PAGE proteins was considerable. No isolate possessed identical combinations of the various alleles of each protein. Six allelic forms of protein C, 7 of protein D, 7 of protein K and 2 each of proteins G, L, F, N and P were detected. All the possible combina-

tions of the allelic variants of proteins N, L and F were found. Associations between the variants of these proteins were examined by χ^2 tests between each pair of loci. No evidence of linkage between any of these loci was found at a significance level of $P < 0.05$ (Table 3).

Previous work has established that protein P is the enzyme ADA (FENTON & WALLIKER, 1990). Variant forms of ADA and of protein P were correlated as expected (Table 1).

(iii) *Antigens*. Eight allelic serotypes (corresponding to different allelic variants of the MSA-1 gene), recognized by specific Mab combinations, were detected in the village. Serotypes 49 and 50, recognized by Mab 6.1, were found in more than 40% of isolates. Mixed infections of parasites with different MSA-1 alleles were identified in 10 patients. Four allelic serotypes of MSA-2 were identified. The epitope on the Exp-1 antigen recognized by Mab 5.1 was present in 45% of the isolates.

Discussion

The work reported in this and the accompanying paper (BABIKER *et al.*, 1991) is the first detailed genetic characterization of the *P. falciparum* population occurring in a small community. The most significant finding was the remarkable degree of polymorphism in these parasites. At least 2 alleles of each of the genes investigated were found, and each of the 29 patients examined during a two-week period contained parasites of different genotypes. The findings reinforce those of CREASEY *et al.* (1990), who showed that no 2 isolates in a collection of 60 from different regions of Thailand, Zimbabwe and Brazil were identical. These data, therefore, provide no evidence for the hypothesis of 'clonality' in *P. falciparum*, which predicts that organisms with the same genotype should occur frequently in natural parasite populations (TIBAYRENC *et al.*, 1990).

The diverse forms of the characters we examined have been well documented in previous surveys. For example, the electrophoretic forms of the enzymes used were first described by CARTER & MCGREGOR (1973), CARTER & VOLLER (1975), SANDERSON *et al.* (1981) and THAITHONG *et al.* (1981). The frequencies of the forms of GPI and ADA accord with those described from other African countries. The discovery of three forms of PEP in a single village is of interest. PEP-1 is by far the most common form of this enzyme in most countries, with the exception of Tanzania, where 6 of 8 isolates examined by SANDERSON *et al.* (1981) were PEP-2 and 2 were PEP-3. The presence of forms 2 and 3 in Asar suggests that these

Table 3. Association between alleles of proteins L, N and F in isolates of *P. falciparum* from Asar^a

(i) Proteins L and N		
Protein	N-1	N-2
L-1	4 (4)	4 (3.5)
L-2	6 (5)	5 (5)
(ii) Proteins F and N		
Protein	N-1	N-2
F-1	4 (3.6)	8 (6.5)
F-2	4 (3)	6 (5.4)
(iii) Proteins L and F		
Protein	F-1	F-2
L-1	5 (7.2)	9 (6.8)
L-3	13 (10.8)	8 (10.0)

^a χ^2 tests were performed on the 3 data sets. Figures in brackets are the expected values on the basis of free association between alleles at each locus (i.e., unlinked). The test for proteins L and F was significant at $P < 0.01$. However, since 3 pairwise comparisons were performed, the conventional significance value of $P = 0.05$ should be reduced by a factor of 3, i.e. to $P = 0.017$, to eliminate spurious positive association; at this value, none of the associations was positive.

forms are particularly common in East Africa.

Among the 2D-PAGE proteins, forms of D, F, G, I, N and P occurred at similar frequencies to those seen in other countries, especially Zimbabwe (CREASEY *et al.*, 1990). Forms of protein C were difficult to detect in 14 of the samples examined. Among the isolates in which it could be found, C10 is a new allele reported here for the first time. C2, present in 4 isolates, had previously been found only in Thailand. C3 and C9, detectable in other isolates of Asar, have previously been found only in African countries. Considerable variation was also found in protein K, as seen elsewhere (CREASEY *et al.*, 1990); K10 is a previously undescribed form of this protein.

The frequencies of the MSA-1 and MSA-2 alleles were similar to those found in Zimbabwe. The 6·1 epitope, present on alleles 49 and 50, occurs frequently in Zimbabwe but at low frequency in West Africa (CREASEY *et al.*, 1990; CONWAY & MCBRIDE, 1991). The 5·1 epitope of antigen Exp-1, found in 14 of the isolates we examined, has also been found at a high frequency (72%) in Zimbabwe (CREASEY *et al.*, 1990). It is possible that host-parasite interactions may be responsible for the diversity seen in antigens such as MSA-1 and MSA-2. Frequencies of alleles of these antigens were stable over time in an urban/peri-urban area of The Gambia (CONWAY & MCBRIDE, 1991). On the other hand, FORSYTH *et al.* (1989) showed differences in the prevalence of an S-antigen allelic serotype between villages in Papua New Guinea, and within the same village over time.

Mixed infections with more than one parasite genotype were detected in 52% of patients in Asar and this percentage would clearly increase in proportion to the sensitivity of detection methods and the number of loci surveyed. Similar observation of multi-clonal infections have been made by others (e.g. CARTER & MCGREGOR, 1973; THAITHONG *et al.*, 1984; LOCKYER *et al.*, 1989; CREASEY *et al.*, 1990; CONWAY *et al.*, 1991). The precise genotypes in mixed isolates can normally be identified only by cloning. However, it seems clear from the surveys of uncloned isolates that genetically identical *P. falciparum* clones are rarely found. The results from the 2 household pairs showed that even in circumstances where infections could have been contracted at the same time, the parasites in each patient were different. Such infections might have been initiated by 2 or more mosquitoes infected with genetically different sporozoites, or by different sporozoites from the same mosquito.

The origins of the diversity in the characters studied here, and the mechanisms by which they are maintained, are not understood. Asar is a comparatively small village in an area of seasonal malaria transmission, and thus a limited gene pool might have been expected in its population. There is, however, some movement of the population between Asar, neighbouring villages, and the town of Gedaref in which *P. falciparum* is common during the same season. Refugees from Ethiopia have also passed through, or settled in, the region, and infections imported by such people could account for the diversity of parasite types.

The diversity of parasite genotypes seen in this study can be explained most satisfactorily as a result of recombination between the loci for the genes involved. Pairwise comparisons between the 2D-

PAGE proteins L-N, L-F and N-F (Table 3) showed that all possible combinations of the different alleles of each gene pair occurred. While the number of isolates tested was small, there was no evidence of linkage disequilibrium; the alleles assorted independently, presumably due to random recombination events during meiosis. A genetic cross between 2 genetically distinct *P. falciparum* clones generated progeny clones in which recombination had occurred between all the protein variants and enzymes used in the study (WALLIKER *et al.*, 1987; FENTON & WALLIKER, 1990). This study also demonstrated that recombinant parasites may be generated at a higher than expected frequency. We consider, therefore, that a non-recombining clonal population structure for *Plasmodium* parasites in nature, such as that proposed by TIBAYRENC *et al.* (1990), is not applicable here. In the parasite population in Asar, a high frequency of cross-fertilization between clones in mosquitoes, followed by genetic recombination between polymorphic gene loci at meiosis, most probably accounts for the remarkable heterogeneity of the *P. falciparum* in the village.

Acknowledgements

We acknowledge with gratitude continuing support from the Medical Research Council of Great Britain, The Wellcome Trust, and the World Health Organization (TDR). Richard Fawcett provided expert technical assistance in culturing parasites. Field studies in Sudan are generously funded by grants from the National Institutes of Health, USA (AI-16312) and the Danish Agency for International Development (DANIDA). We particularly thank Dr James Jensen for valuable assistance and encouragement. Hamza Babiker is supported by funds from the Overseas Development Administration of Great Britain, administered by the British Council. The work would not have been possible without the help of the Malaria Administration of Sudan. We are also happy to acknowledge the goodwill and excellent co-operation of the staff of Gedaref Hospital and the people of Asar.

References

- Babiker, H. A., Creasey, A. M., Bayoumi, R. A. L., Walliker, D. & Arnot, D. E. (1991). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the *mdr1* genotype of recent isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **85**.
- Bayoumi, R. A., Babiker, H. A., Ibrahim, S. M., Galib, H. W., Saeed, B. O., Khider, S. & Karim, E. A. (1989). Spread of chloroquine resistant *Plasmodium falciparum* in Sudan. *Acta Tropica*, **46**, 157-165.
- Carter, R. & McGregor, I. A. (1973). Enzyme variation in *Plasmodium falciparum* in The Gambia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **73**, 830-837.
- Carter, R. & Voller, A. (1975). The distribution of enzyme variation in populations of *Plasmodium falciparum* in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **69**, 371-376.
- Carter, R., Graves, P. M., Creasey, A., Byrne, K., Read, D., Alano, P. & Fenton, B. (1989). *Plasmodium falciparum*: an abundant stage-specific protein expressed during early gametocyte development. *Experimental Parasitology*, **69**, 140-149.
- Conway, D. J. & McBride, J. S. (1991). Population genetics of *Plasmodium falciparum* within a malaria hyperendemic area. *Parasitology*, in press.
- Conway, D. J., Greenwood, B. M. & McBride, J. S. (1991). The epidemiology of multiple-clone *Plasmodium falciparum* infections in Gambian patients. *Parasitology*, in press.

- Creasey, A., Fenton, B., Walker, A., Thaithong, S., Oliveira, S., Mutambu, S. & Walliker, D. (1990). Genetic diversity of *Plasmodium falciparum* shows geographical variation. *American Journal of Tropical Medicine and Hygiene*, **42**, 403-413.
- El Gaddal, A. (1986). Malaria in the Sudan. In: *Proceedings of the Conference on Malaria in Africa*, Buck, A. A. (editor). Washington: American Institute of Biological Sciences, pp. 156-159.
- Fenton, B. (1987). Peptide digest studies of polymorphic proteins of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **24**, 13-22.
- Fenton, B. & Walliker, D. (1990). Genetic analysis of polymorphic proteins of the human malaria parasite *Plasmodium falciparum*. *Genetical Research*, **55**, 81-86.
- Fenton, B., Walker, A. & Walliker, D. (1985). Protein variation in clones of *Plasmodium falciparum* detected by two dimensional electrophoresis. *Molecular and Biochemical Parasitology*, **16**, 173-183.
- Fenton, B., Clark, J. T., Wilson, C. F., McBride, J. S. & Walliker, D. (1989). Polymorphism of 35-48 kDa *Plasmodium falciparum* merozoite surface antigen. *Molecular and Biochemical Parasitology*, **34**, 79-86.
- Forsyth, K. P., Anders, R. F., Cattani, J. A. & Alpers, M. P. (1989). Small area variation in prevalence of an S-antigen serotype of *Plasmodium falciparum* in villages of Madang, Papua New Guinea. *American Journal of Tropical Medicine and Hygiene*, **40**, 344-350.
- Kemp, D. J., Coppel, R. L., Stahl, H. D., Bianco, A. E., Corcoran, L. M., McIntyre, P., Langford, C. J., Favaloro, J. M., Crewther, P. E., Brown, G. V., Mitchell, G. F., Culvenor, J. G. & Anders, R. F. (1986). Genes for antigens of *Plasmodium falciparum*. *Parasitology*, **91**, supplement, S83-S108.
- Kemp, D. J., Cowman, A. & Walliker, D. (1990). Genetic diversity in *Plasmodium falciparum*. *Advances in Parasitology*, **29**, 75-149.
- Lockyer, M. J., Marsh, K. & Newbold, C. (1989). Wild isolates of *Plasmodium falciparum* show extensive polymorphism in T cell epitopes of the circumsporozoite protein. *Molecular and Biochemical Parasitology*, **37**, 275-280.
- McBride, J. S., Walliker, D. & Morgan, G. (1982). Antigenic diversity in the human malaria parasite *Plasmodium falciparum*. *Science*, **217**, 254-257.
- McBride, J. S., Newbold, C. I. & Anand, R. (1985). Polymorphism of a high molecular weight schizont antigen of the human malaria parasite *Plasmodium falciparum*. *Journal of Experimental Medicine*, **161**, 160-180.
- Omer, A. H. S. (1978). Species prevalence of malaria in northern and southern Sudan, and control by mass chemo-prophylaxis. *American Journal of Tropical Medicine and Hygiene*, **27**, 858-863.
- Peters, W. (1987). *Chemotherapy and Drug Resistance in Malaria*. London: Academic Press.
- Sanderson, A., Walliker, D. & Molez, J.-F. (1981). Enzyme typing of *Plasmodium falciparum* from some African and other old world countries. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **75**, 263-267.
- Simmons, D., Woollett, G., Bergin-Cartwright, M., Kay, D. & Scaife, J. (1987). A malaria protein exported into a new compartment within the host erythrocyte. *EMBO Journal*, **6**, 485-491.
- Sudan (1986). *Annual Statistical Report*. Khartoum: Ministry of Health.
- Thaithong, S., Sueblinwong, T. & Beale, G. (1981). Enzyme typing of some isolates of *Plasmodium falciparum* from Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **75**, 268-270.
- Thaithong, S., Beale, G. H., Fenton, B., McBride, J., Rosario, V., Walker, A. & Walliker, D. (1984). Clonal diversity in a single isolate of the malaria parasite *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **78**, 242-245.
- Tibayrenc, M., Kjellberg, F. & Ayala, F. J. (1990). A clonal theory of parasitic protozoa: the population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas* and *Trypanosoma* and their medical and taxonomical consequences. *Proceedings of the National Academy of Sciences of the USA*, **87**, 2414-2418.
- Trager, W. & Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science*, **193**, 673-675.
- Trenholme, K. & Phillips, R. S. (1989). The use of murine feeder cells in the cultivation of *Plasmodium falciparum* asexual blood stages. *Parasitology Research*, **75**, 518-521.
- Walliker, D., Quakyi, I. A., Wellems, T. E., McCutchan, T. F., Szarfman, A., London, W. T., Corcoran, L. M., Burkot, T. R. & Carter, R. (1987). Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science*, **236**, 1661-1666.

Received 25 March 1991; accepted for publication 12 April 1991

Announcement

CORPORATE MEMBERSHIP WITH THE COMMONWEALTH TRUST 18 NORTHUMBERLAND AVENUE, LONDON, WC2 5BJ

The Society has Corporate Membership with the Trust, which has premises close to Trafalgar Square. **Overseas Fellows** only can use the facilities of the Trust, when in London, for a period of 21 days in any three months, **provided that all booking is done through the Secretary at Manson House**. Fellows who wish to use these facilities must give the Secretary adequate notice of their requirements particularly during the summer when at least two months' notice will be required. Details of the facilities available will be sent to Fellows on request.

Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the *mdr1* genotype of recent isolates

Hamza Ali Babiker^{1,2}, Alison M. Creasey¹, Riad A. L. Bayoumi³, David Walliker¹ and David E. Arnot¹ ¹*Institute of Cell, Animal and Population Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JN, Scotland, UK;* ²*Malaria Administration, Ministry of Health, Khartoum, Sudan;* ³*Department of Biochemistry, Faculty of Medicine, University of Khartoum, Sudan*

Abstract

Isolates of *Plasmodium falciparum* from a Sudanese village have been collected as part of a study of parasite genetic diversity during seasonal malaria epidemics. The sensitivity *in vitro* to chloroquine, pyrimethamine and mefloquine of these isolates has been determined. To assess the utility of pulse field gel chromosome separations in isolate characterization, 18 samples from individual patients in a single village were studied using this technique. Extensive variation in chromosome size was detected, no 2 isolates having identical molecular karyotypes. No multidrug resistance (*mdr*) gene amplification polymorphisms were detected in either chloroquine-resistant or chloroquine-sensitive isolates in this sample.

Introduction

We are interested in the extent of genetic diversity in the *Plasmodium falciparum* population during seasonal epidemics of malaria in Sudan as a basis for developing an understanding of the factors affecting the population genetics of the parasite during epidemics. A potentially major influence on the genetic composition of *P. falciparum* populations is the spread of drug-resistant, particularly chloroquine-resistant, genotypes under the selection pressure of drug prophylaxis and treatment. Chloroquine resistance was first noted in Sudan in 1987 (BAYOUMI *et al.*, 1989) and has now become a major public health problem. In this study we describe *in vitro* sensitivity profiles of 29 isolates of *P. falciparum* from individuals attending a malaria clinic in the village of Asar in the eastern province of Sudan. Chromosome separations using pulse field gel electrophoresis (SCHWARTZ & CANTOR, 1984) were carried out on material from cultures *in vitro* of 18 of the Asar strains and on a number of isolates from patients attending a clinic in Gedaref hospital, 18 km from Asar. As part of a search for genotypic markers for chloroquine resistance, Southern blots of karyotype separations were hybridized with a *P. falciparum mdr1* gene probe and screened for *mdr1* gene amplifications, which have been reported to be associated with chloroquine resistance *in vitro* (FOOTE *et al.*, 1989; WILSON *et al.*, 1989).

Materials and Methods

Collection of isolates

Infected blood was taken (with informed consent) from residents of Asar village, eastern Sudan, who presented with acute *P. falciparum* malaria at a rural

clinic in Asar village in October–December 1989, during a season of intense malaria transmission in that region. Blood was collected in heparinized Vacutainers® and the patients then started a course of chloroquine. Cultures were set up as described in the preceding paper (BABIKER *et al.*, 1991).

Drug tests

The minimum inhibitory concentration (MIC) of drug which killed all, or nearly all (99%), of the isolates *in vitro* was determined for chloroquine (Nivaquine®, chloroquine sulphate, May and Baker), pyrimethamine (Wellcome) and mefloquine (Roche). A sample from each isolate was tested for drug response by the method described by THAITHONG *et al.* (1983), in which unsynchronized parasites were exposed to varying concentrations of drug in complete RPMI-1640 medium (including 10% serum) for 72 h. Samples were then examined microscopically in thin Giemsa-stained films. Parasitaemia in the starting cultures was between 0.3 and 0.9%. In this test, MIC over a 72 h culture period gives the actual drug concentration needed to kill almost all parasites. In contrast, the World Health Organization 24 h test kit measures growth inhibition. The effective concentration needed to kill 99% of parasites (EC₉₉) is then obtained by extrapolation from a regression line.

Preparation of chromosomal deoxyribonucleic acid (DNA) in agarose blocks

Parasites were generally prepared from 5 ml of asynchronous culture at c. 5% haematocrit and 10% parasitaemia. Parasitized red blood cells were pelleted by centrifugation at 2000 g for 10 min at 4°C. Parasites were released from the red blood cell pellet by lysis with 5 volumes of 0.1% saponin in phosphate-buffered saline (PBS). After incubation at room temperature for 10 min the parasites were pelleted by centrifugation at 4000 g for 15 min. The supernatant was removed and the pellet resuspended in 2 volumes of PBS at room temperature. The suspension was fixed by the addition of 2% low-gelling-temperature agarose in PBS at 45°C to a final agarose concentration of 1.2%. The mixture was put into 1×6×10 mm moulds and allowed to gel at 4°C for 20 min. Agarose blocks were transferred from the mould into 1% sarkosyl, 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 9.0) containing 0.25 mg/ml proteinase K (Boehringer) and incubated for 48 h at 50°C with one buffer change. Treated blocks were stored until required at 4°C in 0.5 M EDTA (pH 9.0). Samples prepared in Sudan directly from human blood infec-

tions were treated in essentially the same fashion except that human leucocytes and platelets were removed by passing heparinized blood samples over an acid-washed glass beads column followed by a CF11 cellulose column. Best results were obtained by culturing human samples for 24 h to allow the development of multinucleate schizonts before preparing chromosome blocks.

Electrophoresis conditions

Pulsed field gel electrophoresis (PFG) was performed in a Bio-Rad CHEF[®] gel apparatus in 6 mm thick 0.8% agarose gels (SeaKem GTG[®] or IBI[®] agarose). Chromosome blocks were placed in pre-formed wells and were sealed in the gels with 1% low-melting-point agarose. The chamber buffer was 0.5×TBE (1×TBE is 90 mM Tris HCl/90 mM boric acid/2.5 mM EDTA) circulated at a constant 12°C. Pulsing times and voltages are described in the relevant figures. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed using ultra-violet transillumination.

Southern blotting and DNA probe hybridization

After photography, chromosomal DNA was cleaved with 0.25 N NaOH for 25 min and transferred to Hybond N⁺[®] nylon membranes (Amersham) according to the manufacturers' alkali transfer protocol. A 4.2 kilobase insert containing the entire *Pfmdr1* gene (a gift from Dr Alan Cowman) was purified from the pIC20 plasmid vector by restriction enzyme digestion followed by agarose gel separation and purification of the relevant band using Gene-Clean[®] (Bio-101). Radiolabelling of the DNA probe was carried out using a random priming labelling kit (Boehringer-Mannheim) and hybridized to the membrane-bound DNA overnight at 42°C in 0.9M NaCl/90 mM sodium citrate, 50 mM sodium phosphate (pH 6.4), 5×Denhardt's solution, 10% dextran sulphate, 0.1% sodium dodecyl sulphate (SDS) and 38% formamide. Filters were washed for 4 h at 42°C with 6 changes of 2×standard saline citrate (SSC), 50 mM sodium phosphate (pH 6.4) and 0.1% SDS.

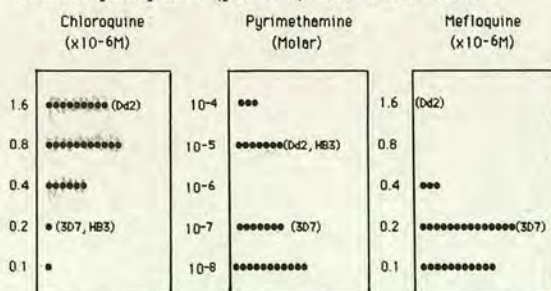


Fig. 1. Drug susceptibility (minimum inhibitory concentrations) of *P. falciparum* from Asar village during October and November 1989. Parasites were incubated in 0.1 ml volumes in microtitre plate wells with drug concentrations successively doubling from 1×10^{-7} M to 1.6×10^{-6} M. For chloroquine and mefloquine these concentrations correspond to microtitre plate layouts with the drug amounts in the 0.1 ml sample wells progressively doubling from 1 µM to 16 µM. For pyrimethamine the sample well drug concentrations increase by a factor of 10 from 10^{-9} M to 10^{-5} M in 5 steps. Each dot represents an Asar 1989 isolate named in the text. *P. falciparum* clones Dd2, 3D7 and HB3 were laboratory strains frequently used in studies of drug resistance (Wellems *et al.*, 1990), utilized in this study as resistance and susceptibility controls.

Results

The study area of Asar village in the Gedaref region of eastern Sudan and the collection and culture methodology used in the study have been described in the preceding paper (BABIKEER *et al.*, 1991).

Fig. 1 and the Table illustrate the response of 28 of the isolates from Asar to the drugs chloroquine, pyrimethamine and mefloquine. There was considerable variation in response to chloroquine and pyrimethamine, but less to mefloquine.

The MIC values for chloroquine for most of the isolates were high, similar to or approaching that of the control resistant clone Dd2; only 2 isolates had similar or lower MICs than those of the sensitive control clones 3D7 and HB3. The remaining isolates had an intermediate response, with MICs which fell between these 2 extremes. There appeared to be 2 levels of response to pyrimethamine. Ten isolates required 10^{-5} M or higher to kill the parasites, a level

Table. Drug responses *in vitro* of isolates of *Plasmodium falciparum* from patients in Asar village, eastern Sudan, during the October–November malaria season of 1989^a

Isolate	Chloroquine ^b ($\times 10^{-6}$ M)	Pyrimethamine ^b (Molar)	Mefloquine ^b ($\times 10^{-6}$ M)
101/89	4	1	2
102/89	5	2	2
104/89	3	1	2
105/89	4	2	1
106/89	4	5	2
107/89	3	2	1
108/89	4	1	2
109/89	1	1	4
110/89	5	5	1
111/89	4	5	2
112/89	5	2	1
113/89	3	4	1
114/89	3	4	2
115/89	2	1	4
116/89	4	1	2
117/89	4	2	2
119/89	5	1	2
120/89	3	2	1
121/89	5	4	2
122/89	5	2	1
123/89	4	4	1
124/89	5	4	2
125/89	5	4	1
126/89	4	1	2
127/89	4	1	1
128/89	3	1	4
129/89	5	1	2
130/89	4	4	1

^aAlthough complete compliance and clinical outcome data were not obtained, most patients responded to the standard course of chloroquine therapy and their parasitaemias were cleared. Although not the only cases of resistance *in vivo*, 4 patients (nos 106, 110, 117 and 122) failed to clear their parasites despite chloroquine treatment (RII and RIII levels of resistance). Isolates from these patients, collected before drug administration, all showed chloroquine resistance *in vivo*.

^bNumbers indicate the minimum inhibitory concentrations of the drugs which killed all, or nearly all, the parasites, as follows. Chloroquine: $1 = 0.1 \times 10^{-6}$ M (sensitive), $2 = 0.2 \times 10^{-6}$ M, $3 = 0.4 \times 10^{-6}$ M, $4 = 0.8 \times 10^{-6}$ M, $5 = 1.6 \times 10^{-6}$ M (resistant); pyrimethamine: $1 = 10^{-8}$ M (sensitive), $2 = 10^{-7}$ M, $3 = 10^{-6}$ M, $4 = 10^{-5}$ M, $5 = 10^{-4}$ M (resistant); mefloquine: $1 = 0.1 \times 10^{-6}$ M (sensitive), $2 = 0.2 \times 10^{-6}$ M, $3 = 0.4 \times 10^{-6}$ M, $4 = 0.8 \times 10^{-6}$ M, $5 = 1.6 \times 10^{-6}$ M (resistant).

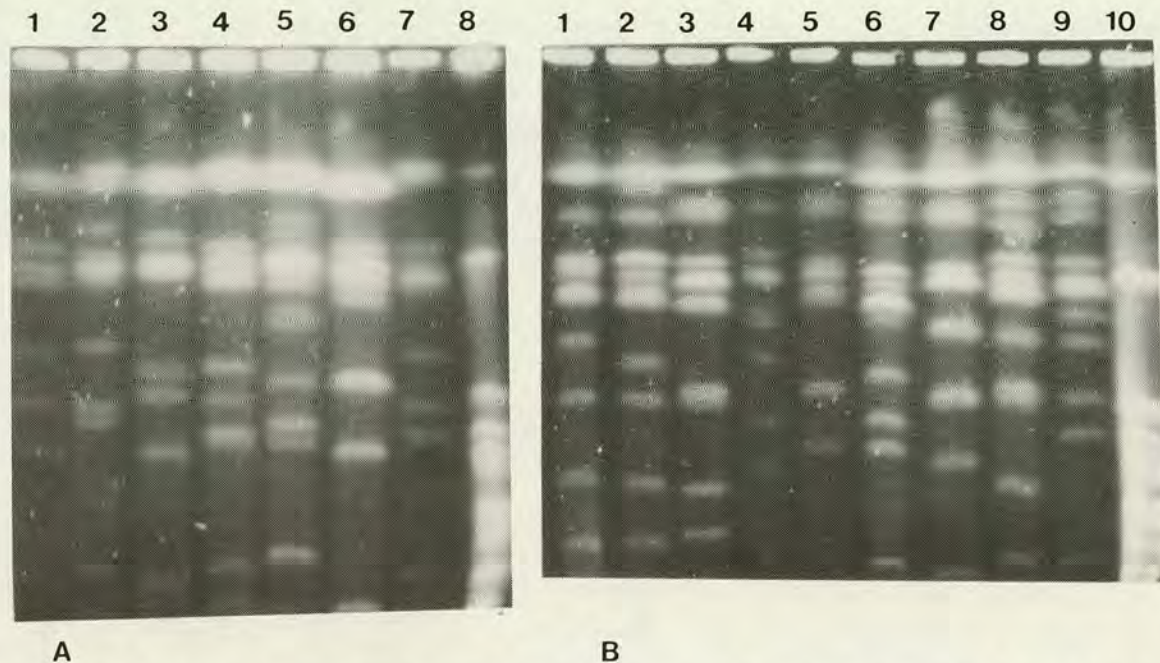


Fig. 2. CHEF[®] pulsed field gels of recent (October–December 1989) isolates of *P. falciparum* from Asar village, eastern Sudan. A. Lane 1: Gedaref 1896/89; lane 2: Asar 129/89; lane 3: Asar 126/89; lane 4: Asar 125/89; lane 5: Asar 123/89; lane 6: Asar 121/89; lane 7: Asar 120/89; lane 8: yeast strain YNN295 markers. Electrophoresis was carried out in 0.8% agarose at a constant 120V with pulse times of 120 s for 20 h, 180 s for 48 h and 300 s for 22 h. B. Lane 1: Thai T9/94; lane 2: Thai T9/96; lane 3: Asar 116/89; lane 4: Asar 115/89; lane 5: Asar 113/89; lane 6: Asar 108/89; lane 7: Asar 106/89; lane 8: Asar 121/89; lane 9: Asar 101/89; lane 10: yeast strain YNN295 markers. Electrophoresis was carried out for 18 h with a pulse time of 120 s at 150V, 24 h of 180 s pulse frequency at 140V, and 18 h of 300 s pulses at 140V. Gedaref 1896/89 was not cultured; chromosome blocks were prepared directly from a patient's blood sample. The 2 Thai samples were cloned isolates. The sizes of the 4 largest yeast chromosome markers in A have been estimated to be 2200, 1600, 1125 and 1020 kilobases respectively. The 5 smallest yeast chromosomes visible had an estimated size of 370, 460, 580, 630 and 700 kilobases (ANAND *et al.*, 1989).

similar to the resistant controls. The remaining 18 isolates were killed at concentrations of 10^{-7} M or less, similar to the sensitive control 3D7. Each of the 28 isolates appeared to be sensitive to mefloquine, all parasites being killed at concentrations of 4×10^{-6} M or less (Fig. 1).

There was no correlation between response to chloroquine and response to pyrimethamine, since all possible combinations of resistance and sensitivity to the 2 drugs were found among the isolates of this village. For example, isolate 110/89 was resistant to both drugs, isolate 107/89 was sensitive to both drugs, isolate 112/89 was resistant to chloroquine and sensitive to pyrimethamine, and isolate 113/89 was resistant to pyrimethamine and sensitive to chloroquine. Although all isolates appeared to be sensitive to mefloquine, there was, again, no relationship between the level of sensitivity and the response to the other 2 drugs (Table).

Molecular karyotype

The molecular karyotypes of 12 *P. falciparum* isolates are shown in Fig. 2. One lane (Fig. 2 A, lane 1) shows a karyotype derived directly from patient's blood without culture *in vitro*. This karyotype therefore represented ring-stage, uninucleate parasites. Unless the patient's parasitaemia is high (at least 3–5%), the amount of parasite DNA in 5 ml samples

of infected blood is too low to permit detection of chromosome bands on stained PFG gels. Two lanes (Fig. 2 B, lanes 1 and 2) illustrate cloned lines from Thai isolates of *P. falciparum* (a gift from Dr Sodsri Thaithong). Nine to 11 chromosome bands were resolved in each of these isolates; under optimal conditions of electrophoresis, up to 14 of these chromosomes can be resolved in *P. falciparum* (WEL-LEMS *et al.*, 1987). While not all isolates showed more bands than the Thai clones, it was clear that many of the uncloned Sudanese isolates did have more and less evenly stained chromosomes, particularly in the 1200–2000 kilobase size range. This multiplicity of unevenly staining bands probably indicated mixed parasite populations within the isolate. In fact, the isolates which showed fewer and more stoichiometrically staining chromosomes frequently corresponded to those judged to be monoclonal on other criteria (BABIKE *et al.*, 1991). It was obvious that each chromosome showed considerable variation in size in this parasite population, and no two isolates were identical. A further 6 Asar isolates have been analysed by PFG (data not shown), without any identical karyotypes being found.

Hybridization of *Pfmdr1* probes to PFG chromosome separation

The PFG separation of 4 Asar isolates of *P.*

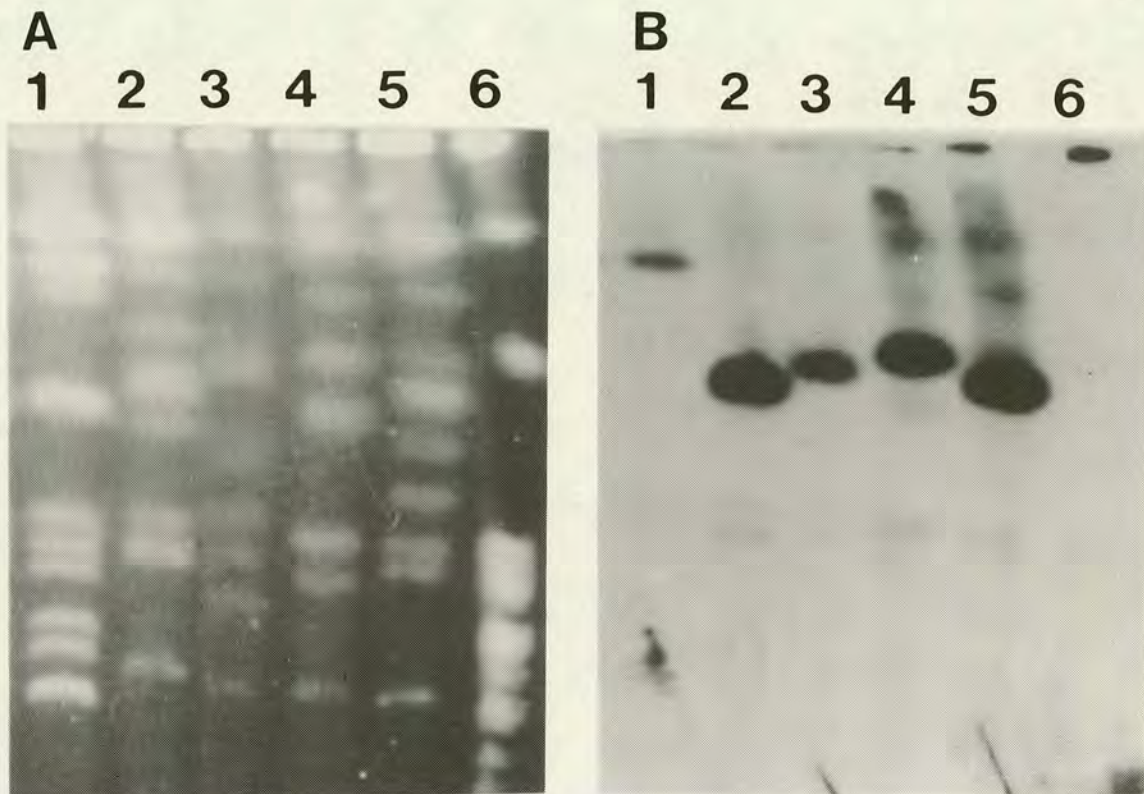


Fig. 3. A. CHEF® pulsed field gel of *P. chabaudi*, and of *P. falciparum* isolates from Asar. Lane 1: *P. chabaudi* AS3CQ clone; lane 2: Asar 101/89; lane 3: Asar 113/89; lane 4: Asar 115/89; lane 5: Asar 129/89; lane 6: yeast strain YNN295 markers (approximate sizes given in legend to Fig. 1). Electrophoresis was carried out in 0.8% agarose at a constant 200V and pulse time of 100 s for 23 h. B. Chromosome separations after transfer to Hybond-N® membranes and hybridization with a ³²P-labelled *P. falciparum* *mdr1* gene probe. The probe hybridizes to chromosome 5 of *P. falciparum* and cross hybridizes to a *P. chabaudi* homologue on chromosome 11 or 12.

falciparum, each with a different level of chloroquine sensitivity, is shown in Figure 3 A. Asar isolates 115/89, 113/89, 101/89 and 129/89 had MIC values of 2×10^{-7} M, 4×10^{-7} M, 8×10^{-7} M and 1.6×10^{-6} M chloroquine respectively. In isolates 101, 113 and 115, the probe appeared to hybridize to chromosome 5, in agreement with the results of FOOTE *et al.* (1989). Hybridization appeared to be to the sixth chromosome (numbered in increasing order of size) in isolate 129 but, given the mixed nature of the parasite population, the extensive chromosome size polymorphisms, and the technical difficulties in resolving the group of *P. falciparum* chromosomes in this region of the gel, it is probable that the gene was on the same chromosome in this isolate. This pattern of hybridization of the *mdr1* gene probe to a single chromosome of a size varying from around 1300 to 1600 kilobases has been repeated for the 18 Asar isolates and 6 Gedaref hospital isolates (data not shown). The results given here are entirely representative of the larger sample. In one sample (Asar 128/89) 2 distinct cross-hybridizing bands were resolved in the 1300–1600 kilobase region. This isolate is known from other markers to be a mixed isolate (see BABIKER *et al.*, 1991) and it presumably contained 2 types of parasite with different sizes of chromosome 5. In no isolate did we

observe any large increase in the size of the chromosome band hybridizing to the *Pfmdr1* probe, nor did we detect any increase or decrease in the intensity of the hybridization signal which was not in proportion to the amount of chromosomal DNA on the gel, as assayed by eye or by cross hybridization with a known single copy gene probe (the dihydrofolate reductase gene).

Discussion

The high frequency of phenotypically chloroquine-resistant isolates in Asar village is not surprising since chloroquine resistance and chloroquine treatment failures were reported from this region of eastern Sudan in 1987 (BAYOUMI *et al.*, 1989). It is probable that chloroquine-resistant *P. falciparum* genotypes are continuing to spread at the expense of sensitive forms, although this process can be confirmed only by continued monitoring, a study which is now going on. Since these isolates contained different parasite genotypes, the absolute levels and range of chloroquine sensitivity in the *P. falciparum* population in this region cannot be measured until clones have been isolated from these mixed populations, a process under way in this laboratory. Pyrimethamine resistance has not been previously reported from this

region and its significance and origin are unclear. Pyrimethamine/sulphadoxine (Fansidar®) combinations constitute the only widely available drug in Sudan in the event of chloroquine treatment failure. The presence of all possible combinations of response *in vitro* to chloroquine and pyrimethamine and the high level of sensitivity to mefloquine suggest that in 'wild' populations resistance to each drug arises by independent mechanisms. There is good evidence that pyrimethamine resistance is due to point mutations in the *P. falciparum* dihydrofolate reductase gene (PETERSON *et al.*, 1988; COWMAN *et al.*, 1988; ZOLG *et al.*, 1990). It therefore seems unlikely that multi-drug resistance to a variety of different drugs (as has been demonstrated for the mammalian P-glycoprotein drug efflux pump by ENDICOTT & LING, 1989), accounts for the various types of resistance seen in Asar.

The molecular karyotypes of *P. falciparum* parasites are known to be remarkably polymorphic (KEMP *et al.*, 1987), with genetically homologous chromosomes having a considerable size range. Chromosome polymorphism in parasites from 5 patients from the Madang area of Papua New Guinea has been reported (CORCORAN *et al.*, 1986) and our results confirmed this finding for a malaria outbreak sampled over a short period within a single village. A remarkable aspect of our results was the extent of parasite diversity in this respect, no 2 isolates sharing an identical karyotype. *P. falciparum* can lose genetic information, especially during long-term culture *in vitro* (POLOGU & RAVETCH, 1986; CORCORAN *et al.*, 1986), although it is likely that chromosome polymorphism is primarily a manifestation of variation in the amount of repetitive DNA associated with generally stable gene linkage groups (KEMP *et al.*, 1987; WELLEMS *et al.*, 1987). This in itself does not explain the extreme karyotype diversity in the Asar *P. falciparum* isolates. Individual chromosomes may grow and shrink independently of each other, as occurs in the chromosome telomeres of *Trypanosoma brucei* (BERNARDS *et al.*, 1982). Chromosomes also exchange DNA segments during crossing over events at meiosis in stages in the mosquito host, as demonstrated for *P. falciparum* by WELLEMS *et al.* (1987) and SINNIS & WELLEMS (1988). It seems likely that frequent recombination accounts for the diversity of genotypes in the parasites of Asar (BABIKER *et al.*, 1991), and it could also account for the extensive chromosomal polymorphisms described here.

The molecular basis of both chloroquine action and parasite resistance to the drug are not well understood and have excited several interesting controversies (WELLEMS *et al.*, 1990; FOOTE *et al.*, 1990; MESHNIK, 1990; GINSBERG, 1990). Resistant strains of *P. falciparum* accumulate less chloroquine than sensitive strains, possibly due to increased efflux of the drug from the resistant parasites (KROGSTAD *et al.*, 1987). Transmembrane pumps hydrolysing adenosine triphosphate are known to extrude structurally diverse drugs in pleiotropically drug-resistant cancer cell lines (ENDICOTT & LING, 1989) and a gene encoding a similar protein (Pfmdr1) has been detected in *P. falciparum* (WILSON *et al.*, 1989; FOOTE *et al.*, 1989). Amplification of this gene has been detected in some, but not all, chloroquine-resistant *P. falciparum* lines. We have not found readily detectable Pfmdr1

gene amplifications in our Asar isolates, despite the chloroquine resistance *in vitro* of a number of our isolates. Our assay does not exclude the possibility that *mdr1* gene over-expression or specific *mdr1* mutations (FOOTE *et al.*, 1990) may correlate with phenotypic chloroquine resistance, although to answer these questions definitively requires the derivation of pure lines from these multi-clonal isolates. However, the results obtained in the present study indicate that *mdr1* gene amplification is not a practical genotypic marker for chloroquine resistance in either epidemiological studies or clinical diagnostic assays. We intend to make use of the permanent nylon membrane-bound chromosome separations of these 1989 Asar isolates to make a retrospective analysis of the spread of drug resistant genotypes, when definitive DNA probes for chloroquine-resistant *P. falciparum* become available.

Acknowledgements

We thank our collaborators Tor Theander, Lars Hviid, James Jensen, Ali Sultan, Bakri Saeed and Gewiria Satti for their help and support in the Sudan Malaria Project. We also thank the Malaria Administration of the Sudanese Ministry of Health for advice and support for this project. This work was supported by the UK Medical Research Council, the Wellcome Trust and USA NIH Grant AI-16312. Hamza Babiker is supported by a British Council Studentship and Riad Bayoumi and David Arnot are Wellcome Travelling Fellow and Senior Biomedical Research Fellow, respectively. We thank the villagers of Asar, Sudan for their support and goodwill.

References

- Anand, R., Villasante, A. & Tyler-Smith, C. (1989). Construction of yeast artificial chromosome libraries with large inserts using fractionation by pulsed-field gel electrophoresis. *Nucleic Acids Research*, **17**, 3425-3433.
- Babiker, H. A., Creasey, A. M., Fenton, B., Bayoumi, R. A., Arnot, D. E. & Walliker, D. (1991). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **85**.
- Bayoumi, R. A., Babiker, H. A., Ibrahim, S. M., Galib, H. W., Saeed, B. O., Khider, S. & Karim, E. A. (1989). Spread of chloroquine resistant *Plasmodium falciparum* in Sudan. *Acta Tropica*, **46**, 157-165.
- Bernards, A., Michels, P. A. M., Lincke, C. R. & Borst, P. (1983). Growth of chromosome ends in multiplying trypanosomes. *Nature*, **303**, 592-597.
- Corcoran, L. M., Forsyth, K. P., Bianco, A. E., Brown, G. V. & Kemp, D. J. (1986). Chromosome size polymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite populations. *Cell*, **44**, 87-95.
- Cowman, A. F., Morry, M. J., Biggs, B. A., Cross, G. A. M. & Foote, S. J. (1988). Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences of the USA*, **85**, 9114-9118.
- Endicott, J. A. & Ling, V. (1989). The biochemistry of P-glycoprotein mediated multidrug resistance. *Annual Reviews of Biochemistry*, **58**, 137-171.
- Foote, S. J., Thompson, J. K., Cowman, A. F. & Kemp, D. J. (1989). Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *Plasmodium falciparum*. *Cell*, **57**, 921-930.
- Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M. J., Forsyth, K., Kemp, D. J. & Cowman, A. F. (1990). Several alleles of the multidrug-resistance gene are

- closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature*, **345**, 255–258.
- Ginsberg, H. (1990). Chloroquine as intercalator: should this hypothesis be revived? *Parasitology Today*, **6**, 230.
- Kemp, D. J., Thompson, J. K., Walliker, D. & Corcoran, L. M. (1987). Molecular karyotype of *Plasmodium falciparum*: conserved linkage groups and expendable histidine-rich protein genes. *Proceedings of the National Academy of Sciences of the USA*, **84**, 7672–7676.
- Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. J., Martin, S. J., Milhous, W. K. & Schlesinger, P. H. (1987). Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science*, **238**, 1283–1285.
- Meshnik, S. (1990). Chloroquine as intercalator: a hypothesis revived. *Parasitology Today*, **6**, 77–79.
- Peterson, D. S., Walliker, D. & Welles, T. E. (1988). Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proceedings of the National Academy of Sciences of the USA*, **85**, 9115–9118.
- Polg, L. G. & Ravetch, J. V. (1986). A chromosome rearrangement in *Plasmodium falciparum* histidine-rich protein gene is associated with the knobless phenotype. *Nature*, **322**, 474–477.
- Schwartz, D. C. & Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*, **37**, 67–75.
- Sinnis, P. & Welles, T. E. (1988). Long range restriction maps of *Plasmodium falciparum* chromosomes: crossing over and size variation among homologous chromosomes from geographically distant isolates. *Genomics*, **3**, 287–295.
- Thaithong, S., Siripoon, N., Seugorn, N., Bunnag, D. & Beale, G. H. (1983). Susceptibility of *Plasmodium falciparum* to five drugs: an *in vitro* study of isolates mainly from Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **77**, 228–231.
- Welles, T. E., Walliker, D., Smith, C. L., Rosario, V. E., Maloy, W. L., Howard, R. J., Carter, R. & McCutchan, T. F. (1987). A histidine-rich protein gene marks a linkage group favoured strongly in a genetic cross of *Plasmodium falciparum*. *Cell*, **49**, 633–642.
- Welles, T. E., Panton, L. J., Gluzman, I. Y., Rosario, V. E., Gwadz, R. W., Walker-Jonah, A. & Krogstad, D. J. (1990). Chloroquine resistance not linked to *mdr*-like genes in *Plasmodium falciparum* cross. *Nature*, **345**, 253–255.
- Wilson, C. M., Serrano, A. E., Walsey, A., Bogenschutz, M. P., Shankar, A. H. & Wirth, D. (1989). Amplification of a gene related to mammalian *mdr* genes in drug resistant *Plasmodium falciparum*. *Science*, **244**, 1184–1186.
- Zolg, J. W., Chen, G.-X. & Plitt, J. R. (1990). Detection of pyrimethamine resistance in *Plasmodium falciparum* by mutation-specific polymerase chain reaction. *Molecular and Biochemical Parasitology*, **39**, 257–267.

Received 25 March 1991; accepted for publication 12 April 1991

Announcement

ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE

Undergraduate Project Prize

The Royal Society of Tropical Medicine and Hygiene offers an annual prize of £200 for an account of work carried out by a non-medical undergraduate student of any nationality. The work will add to the knowledge of human or veterinary health or hygiene in the Tropics. Particular attention in the award of the prize will be directed towards originality and quality. It is intended that the prize should act as a stimulus for the pursuit of excellence in research carried out by undergraduates.

Medical Student Elective Prize

The Royal Society of Tropical Medicine and Hygiene offers an annual prize of £200 for an account of work carried out by a British medical student during an elective period spent in a tropical or developing country. In awarding this prize emphasis will be laid on the originality of the work and on its contribution to knowledge or understanding of tropical diseases.

Further information and advice concerning both prizes can be obtained from the Honorary Secretaries, Royal Society of Tropical Medicine and Hygiene, Manson House, 26 Portland Place, London, W1N 4EY.

MOLBIO 01774

Short Communication

Rapid and simple method for isolating malaria DNA from fingerprick samples of blood

Michael Foley^a, Lisa C. Ranford-Cartwright^a and Hamza A. Babiker^{a,b}

^a*Institute of Cell, Animal and Population Biology, The University of Edinburgh, Edinburgh, UK; and* ^b*Malaria Administration, Ministry of Health, Sudan*

(Received 13 March 1992; accepted 13 March 1992)

Key words: *Plasmodium falciparum*; DNA isolation; Polymerase chain reaction

Epidemiological studies of parasitic diseases are often hampered by the need to isolate parasite DNA before performing PCR. These isolation procedures can be difficult and time consuming, often involving many steps such as the commonly used phenol based extraction procedure. Isolating DNA from many parasite strains or field isolates can be labour intensive and expensive. A simple and rapid procedure for isolating parasite genetic material from a small volume of blood, e.g. a fingerprick sample, would be advantageous. A major limitation is the inhibition of *Taq* polymerase by various substances including haemoglobin. Some recent publications have begun to tackle this problem. Replacement of *Taq* by *Tth* polymerase and the use of anti-histone antibodies have been tried, although the sensitivity of these methods was not fully explored [1,2]. It is important in some genetic studies to detect extremely small parasitaemias in the blood of infected individuals. Another recent advance

has involved dotting blood samples directly onto glass fibre discs and removing haemoglobin by extensive washing [3], although contamination may be one potential problem with this method. We describe in this study a simple and rapid method for preparing enough DNA material from 20 μ l of whole blood or blood frozen in liquid nitrogen without the need for the expensive and time consuming procedure of culturing the parasite. The whole procedure takes only 50 min and is carried out in a single microfuge tube, thus reducing the chances of contamination. No expensive or dangerous chemicals are used, and we have been able to detect down to 77 parasites per μ l of whole blood. Unlike other methods the use of such a small volume of blood makes this method exceptionally valuable for the field operations where venous bleeding of children needs authorised expertise and can be inconvenient. Moreover the detection of low parasitaemias makes this technique very useful for epidemiological studies of parasite populations in the field where low parasitaemias are common.

20 μ l of whole blood (or in vitro culture material) was added to 500 μ l of ice-cold 5 mM sodium phosphate pH 8.0 (5P8), vortexed, and centrifuged in a microfuge for 10 min. The supernatant was discarded and the process repeated twice more. Finally, 50 μ l of sterile

Correspondence address: Michael Foley, Institute of Cell, Animal and Population Biology, The University of Edinburgh, Crew Building, King's Buildings, West Mains Road, Edinburgh, EH9 3JN, UK.

Abbreviations: PCR, polymerase chain reaction; MSP1, merozoite surface protein 1; MSP2, merozoite surface protein 2; RESA, ring infected erythrocyte surface antigen.

water was added to the pellet, vortexed and boiled for 10 min. After centrifugation for 10 min the pellet was discarded and the supernatant used as the starting material for PCR.

If blood samples are fresh we have found that freezing them for 2 min before starting helps in the extraction of DNA.

DNA was amplified by the PCR method [4]. Typically, 1–3 μ l of supernatant was used as a template in a 50 μ l reaction containing 5 μ l 10 \times reaction buffer (100 mM Tris, pH 8.3/500 mM KCl/25 mM MgCl₂), 1 μ l dNTP mix (5 mM each of dATP, dCTP, dTTP, dGTP), 100 pmol of each of the appropriate 5' and 3' primers and 0.4 μ l *Taq* polymerase (AmpliTa_q, 2 units).

Thirty to thirty-five cycles ((95°C/60 s) (45°C/60 s) (68°C/60 s)) were performed on a Biometra TRIO thermoblock. If nested PCR was being carried out, 1 μ l from the first PCR was added to a second tube containing all necessary components plus 2 primers internal to the initial 2, and a further round of 25 cycles performed. 1 μ l of a 1 in 10 dilution of Perfect Match (Stratagene) was added to all PCRs.

We obtained blood from a patient infected with *P. falciparum* malaria and made serial dilutions with whole uninfected blood. After treating 20 μ l samples of each dilution as above and performing PCR with oligos to the gene encoding the malarial protein MSP1 (merozoite surface protein, also called Pf 195) we observed the limits of detection were between 0.01% and 0.005% parasitaemias (Fig. 1a).

Small samples (20 μ l) of blood from infected individuals from Tanzania, frozen in liquid nitrogen, were treated as above and PCR performed using primers to the gene encoding the malarial protein RESA. We were able to detect down to 500 parasites per μ l of blood but not 200 parasites per μ l (Fig. 1b). After PCR using nested primers to the MSP1 gene we were able to detect 200 parasites per μ l of blood. In addition size polymorphisms commonly found in this gene were observed (Fig. 1c). Negative controls consisting of whole uninfected blood were performed with all experiments and failed to produce any bands after PCR.

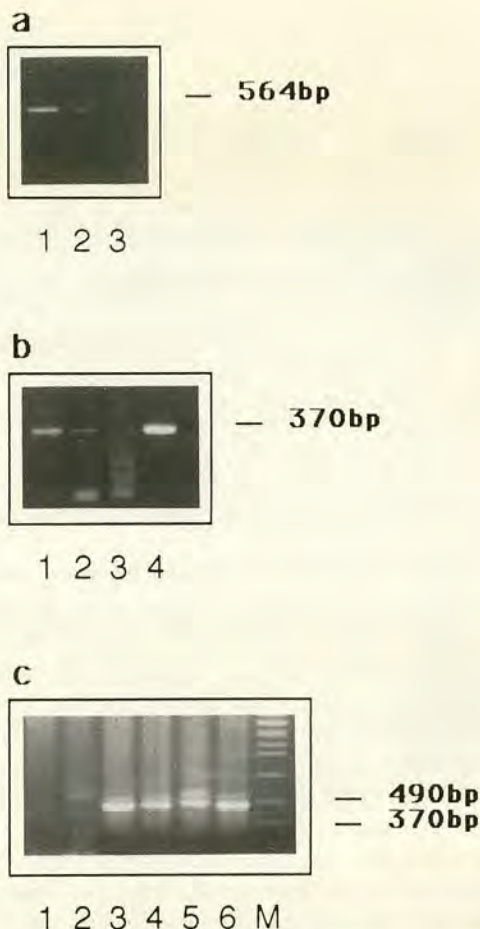


Fig. 1. (a) PCR amplified fragment from individual blood samples with differing parasitaemias using primers to MSP-1. Oligonucleotide primers were synthesised to the conserved regions 1 and 3, (sense (nt 62–88), CACAT-GAAAGTTATCAAGAACTTGTC; antisense (nt 687–707), GTACGTCTAATTCATTGTCAC) [5]. Infected blood was serially diluted with whole uninfected blood to obtain the required concentration of parasites. Lane 1, 0.1%; lane 2, 0.01%; lane 3, 0.001%. Fragments were electrophoresed in 1.8% agarose. (b) PCR-amplified fragments from field samples using primers to the RESA gene of FC27 strain [7] (sense (nt 2996–3011), AATATAT-TACAACCA; antisense (nt 3306–3321), GTTCCGTTA-TCAAC). Calculated numbers of parasites per μ l of blood were: lane 1, 1000; lane 2, 500; lane 3, 200. Lane 4 was a positive control using purified *P. falciparum* DNA. (c) Nested PCR using MSP-1 primers in (a) and 2 internal primers (sense (nt 112–131), GCAGTATTGACAGGTT-ATGG; antisense (nt 661–678), GATTGAAAGGTATTT-GAC). Lane 1: negative control, no DNA. Lane 2: positive control, purified DNA from *P. falciparum* clone 3D7. Lanes 3–6: several field isolates from Tanzania. Note in lane 5 the presence of two bands indicative of a person infected with at least two genotypes of parasite, and the size polymorphism within the studied isolates.



Fig. 2. Nested PCR amplified products from 4 infected individuals in Tanzania using primers to the MSP2 gene from MAD71 strain of *P. falciparum*. First round primers: (sense (nt 3–23), GAAGGTAATTAACATTGTC; antisense (nt 789–811), GAGGGATGTTGCTGCTCCACAG). Second round primers: (sense (nt 111–129), GAGTATAAGGAGAAGTATG; antisense (nt 709–728), CTAGAACCATGAATATGTCC). Lane 1, TIF55 (549 parasites per μ l of blood); lane 2, TIF57 (752 parasites per μ l of blood); lane 3, TIF58 (77 parasites per μ l of blood); lane 4, TIF68 (159 parasites per μ l of blood). Lanes labelled 'm' are DNA size markers (Boehringer, type VI) pBR 328 cut with *Bgl*I and *Hinf*I. The 653-bp fragment is arrowed.

DNA was prepared from the blood of 4 individuals from a village in Tanzania and used as a template in PCR experiments with primers to the merozoite surface protein 2 (MSP2) gene. Two sets of primers which annealed to the conserved termini of the gene were used for nested PCR giving an amplified fragment that spanned the region of the gene shown to be highly polymorphic [6]. By observing the differing sizes of the amplified products we have obtained information on the genetic diversity of natural parasite populations (manuscript in preparation). The results shown in Fig. 2 reveal several interesting features. Our method of rapid DNA isolation can detect an ethidium-stained band from blood from an individual infected with 77 parasites μ l⁻¹ of blood (lane 3), thus reducing the need to detect bands by Southern blotting. It is clear from the figure that individuals infected with multiple parasite genotypes can be detected by the presence of multiple bands representing different alleles of the MSP2 gene (e.g., lane 1). Fig. 2 also reveals the extreme size polymorphism that exists in this molecule.

Longer running of the gel shows that of the 4 individuals examined 6 clearly defined size fragments were observed corresponding to 6 different alleles. Southern blotting of the PCR fragments followed by probing with sequence specific oligonucleotides has confirmed the presence of several alleles. In addition, direct sequencing of PCR bands using these primers has identified at least 2 alleles of MSP1 [5], and one allele of MSP2.

Interestingly, the parasites in the blood from individual TIF68 (lane 4) were found on thick blood film analysis to consist completely of gametocytes.

DNA has been successfully isolated from whole blood stored in liquid nitrogen both with and without 'Glycerolite' frozen packed erythrocytes from infected individuals, samples stored at -20°C , fresh blood from infected individuals and samples from in vitro culture, both frozen and unfrozen.

Haemoglobin, which is known to inhibit *Taq* polymerase, is removed from erythrocytes by incubation and washing in 5P8. We have found that removal of approximately half of the white layer at the top of the tube after centrifugation improves the sensitivity of detection by PCR. Parasite genetic material is released by boiling, which will also serve to inactivate parasite proteases and nucleases. If the material was to be used immediately, a 1–3- μ l sample of supernatant was removed and used as template for PCR. If more studies are to be performed later the supernatant can be separated from the pellet debris transferred to a fresh tube and stored. Supernatants were used successfully in PCR after storage at -20°C for at least 3 months.

Acknowledgements

We would like to thank the Wellcome Trust for supporting this work. Hamza Babiker is supported by the O.D.A. (British Council).

References

- 1 Panaccio, M. and Lew, A. (1991) PCR based diagnosis in the presence of 8% (v/v) blood. *Nucleic Acids Res.* 19, 1151.
- 2 Lew, A. and Kelly, J. (1991) Capture of chromosomal DNA by anti-histone antibodies for PCR. *Nucleic Acids Res.* 19, 3459.
- 3 Kain, K.C. and Lanar, D.E. (1991) Determination of genetic variation within *Plasmodium falciparum* by using enzymatically amplified DNA from filter paper disks impregnated with whole blood. *J. Clin. Microbiol.* 29, 1171–1174.
- 4 Saiki, R.K., Gelfand, D.H., Stoffel, S., Sharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487–491.
- 5 Ranford-Cartwright, L.C., Balfe, P., Carter, R. and Walliker, D. (1991) Direct sequencing of enzymatically amplified DNA of alleles of the merozoite surface antigen MSA-1 gene from the malarial parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 46, 185–188.
- 6 Smythe, J.A., Coppel, R.L., Day, K.P., Martin, R.K., Oduola, A.M.J., Kemp, D.J. and Anders, R.F. (1991) Structural diversity in the *Plasmodium falciparum* merozoite surface antigen 2. *Proc. Natl. Acad. Sci. USA* 88, 1751–1755.
- 7 Favalaro, J.M., Coppel, R.L., Corcoran, L.M., Foote, S.J., Brown, G.V., Anders, R.F. and Kemp, D.J. (1986) Structure of the RESA gene of *Plasmodium falciparum*. *Nucleic Acids Res.* 14, 8265–8277.

threat to health than Edge Hill virus itself. Two monoclonal antibodies, 1B7 and 4G2, which react with epitopes common to Edge Hill and dengue viruses, enhanced infection of a human monocytic cell line (U-937) by dengue virus (HENCHAL *et al.*, 1985). Most isolations of Edge Hill virus have been made in the north-east region of Australia (DOHERTY, 1972) where dengue 1 appears to have become endemic and the first cases of dengue 2 infection for almost 50 years were diagnosed in 1992.

Apart from drawing attention to the possibility of clinical infections with Edge Hill virus, this report raises 2 other significant issues. Diagnostic laboratories which do not routinely test for antibody against Edge Hill virus should be aware of the close serological relationship between it and dengue 2, even in IgM assays which are often virus specific (SCOTT *et al.*, 1972), and consideration may need to be given to the possibility of antibody against Edge Hill virus enhancing subsequent dengue infections in residents of areas where only a single dengue serotype is in circulation.

We thank Drs Madden and Chew for providing clinical data and Jenny Haig and Christine Aitken for excellent technical assistance. This study was supported, in part, by a donation from Dr T. B. Lynch, Rockhampton.

References

- Aaskov, J. G., Ross, P., Davies, C. E. A., Innis, M. D., Guard, R. W., Stallman, N. D. & Tucker, M. (1981). Epidemic polyarthritides in north-eastern Australia, 1978–1979. *Medical Journal of Australia*, **ii**, 17–19.
- Blok, J., Henchal, E. A. & Gorman, B. M. (1984). Comparison of dengue viruses and some other flaviviruses by cDNA-RNA hybridisation analysis and detection of a close relationship between dengue virus serotype 2 and Edge Hill virus. *Journal of General Virology*, **65**, 2173–2181.
- Doherty, R. L. (1972). Arboviruses of Australia. *Australian Veterinary Journal*, **48**, 172–180.
- Doherty, R. L., Marks, E. M., Carley, J. G. & Mackerras, M. J. (1963). Studies of arthropod-borne virus infections in Queensland. III. Isolation and characterisation of virus strains from wild caught mosquitoes in north Queensland. *Australian Journal of Experimental Biology and Medical Science*, **41**, 17–40.
- Doherty, R. L., Carley, J. G., Kay, B. H., Filippich, C., Marks, E. N. & Frazier, C. L. (1979). Isolation of virus strains from mosquitoes collected in Queensland, 1972–1976. *Australian Journal of Experimental Biology and Medical Science*, **57**, 509–520.
- Francis, T. (1955). The current status of the control of influenza. *Annals of Internal Medicine*, **43**, 534–538.
- Hawkes, R. A., Boughton, C. R., Naim, H. M., Wild, J. & Chapman, B. (1985). Arbovirus infections of humans in New South Wales. Sero-epidemiology of the flavivirus group of togaviruses. *Medical Journal of Australia*, **143**, 555–561.
- Henchal, E. A., McCown, J. M., Burke, D. S., Sequin, M. C. & Brandt, W. E. (1985). Epitope analysis of antigenic determinants on the surface of dengue 2 virions using monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene*, **34**, 162–169.
- Hunder, G. G. & Hazleman, B. L. (1985). Giant cell arteritis and polymyalgia rheumatica. In: *Textbook of Rheumatology*, Kelly, W. N., Harris, E. D., Ruddy, S. & Sledge, C. B. (editors), 2nd edition. London: W. B. Saunders, pp. 1166–1173.
- Lumley, G. F. & Taylor, F. H. (1943). *Dengue*. Sydney: School of Public Health and Tropical Medicine, Commonwealth of Australia, Service Publication no. 3.
- Marshall, I. D. & Woodroffe, G. M. (1975). Epidemiology of arboviruses. *Report of the John Curtin School of Medical Research*, p. 2.
- Scott, R. M., McCown, J. M. & Russell, P. K. (1972). Human immunoglobulin specificity after group B arbovirus infections. *Infection and Immunity*, **6**, 277–281.
- Spradbrow, P. B. (1972). Arbovirus infections of domestic animals in Australia. *Australian Veterinary Journal*, **48**, 181–185.
- Wiemers, M. A. & Stallman, N. D. (1975). Immunoglobulin M in Murray Valley encephalitis. *Pathology*, **7**, 187–191.

Received 21 July 1992; accepted for publication 8 October 1992

Announcement

Annual International Course on Identification of Arthropods and other Insects of Medical and Veterinary Importance

18 April–13 May 1994: London, UK

Further information can be obtained from: Dr M. J. R. Hall, Medical and Veterinary Division, Department of Entomology, The Natural History Museum, Cromwell Road, London, SW7 5BD, UK; phone (0)71-938-9451, fax (0)71-938-9395/8937.

Drug response and genetic characterization of *Plasmodium falciparum* clones recently isolated from a Sudanese village

Riad A. L. Bayoumi², Alison M. Creasey¹, Hamza A. Babiker^{1,4}, Jane M.-R. Carlton¹, Ali A. Sultan^{1,3}, Gwiria Satti³, Awinder K. Sohal¹, David Walliker¹, James B. Jensen⁵ and David E. Arnot¹ ¹*Institute of Cell, Animal and Population Biology, University of Edinburgh, Scotland, UK;* ²*Biochemistry Department, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al-Ain, United Arab Emirates;* ³*Faculty of Medicine, University of Khartoum, Khartoum, Sudan;* ⁴*Malaria Administration, Ministry of Health, Khartoum, Sudan;* ⁵*Brigham Young University, Provo, Utah, USA*

Abstract

We have isolated 20 clones of *Plasmodium falciparum* from isolates from patients attending a village clinic in Sudan during 10 d in October–November 1989. The clones were genetically diverse, having highly variable molecular karyotypes and a wide range of drug responses. Chloroquine-sensitive (50% inhibitory concentration [IC₅₀] in the 4–15 nM range) and chloroquine-resistant clones (IC₅₀ in the 40–95 nM range) co-existed in the population, but no obvious amplification of the P-glycoprotein homologue gene, *Pgh1* (previously known as the multi-drug resistance gene, *mdr1*) marked the chloroquine-resistant clones. Chloroquine resistance was reversible by verapamil in these clones, although they varied in their susceptibility to verapamil alone. These observations indicate that the biochemical characteristics of the Sudanese chloroquine-resistant *P. falciparum* are similar to those reported from south-east Asian and Latin American isolates, which is consistent with there being a similar molecular basis for this phenomenon.

Introduction

Chloroquine is the only antimalarial drug available at a cost accessible to even a minority of the Sudanese people and as such constitutes the major element of malaria control in Sudan and similar African countries (PETERS, 1987). The emergence of chloroquine-resistant *Plasmodium falciparum* in the Eastern Province since around 1986 (BAYOUMI *et al.*, 1989) is causing acute problems as the episodic, post-rainy season malaria epidemics characteristic of sub-Saharan Sudan cease to be controlled by this drug.

The mode of action of chloroquine and the mechanism of resistance to the drug are unclear, and several opposing viewpoints exist (HOMEWOOD *et al.*, 1972; KROGSTAD *et al.*, 1987; GINSBURG, 1988; WARHURST, 1988; FOOTE *et al.*, 1990; WELLEMS *et al.*, 1990). Studies *in vitro* on chloroquine resistance in different laboratories have made use of different long-term cultured lines of *P. falciparum* originating mainly from south-east Asia or the Amazon basin. Conclusions drawn from the analysis of such isolates may not accurately reflect the present situation in an area such as Sudan. Although currently spreading rapidly, chloroquine-resistant *P. falciparum* was not reported in Sudan until 25 years after its appearance in south-east Asia and South America, and several years after its appearance in East Africa (AL TAWIL & AKOOD, 1983). Chloroquine is essentially the only antimalarial drug available in most of Sudan, and for economic and climatic reasons usage has not been as heavy as in more prosperous areas with more stable malaria transmission.

In order to study the genetic basis for the spread of chloroquine-resistant *P. falciparum*, we have recently characterized 29 isolates from a single village in eastern Sudan with a developing problem of chloroquine-resistant malaria (BABIKER *et al.*, 1991a, 1991b). Since most of these isolates proved to be mixtures of genetically different parasites, we have now obtained cloned lines from some of the isolates, in order to define parasite genotypes and their biochemical profiles of drug resistance. We showed that the clones had highly variable molecular karyotypes and wide ranges of drug responses. Chloroquine resistance is reversible by verapamil, but no obvious amplification of the P-glycoprotein homologue gene, *Pgh1* (previously known as the multi-drug resistance gene, *mdr1*) marked the chloroquine-resistant clones.

Materials and Methods

Study area

The study area in Asar village, 20 km from Gedaref in the Eastern Province of Sudan, has been described elsewhere (BABIKER *et al.*, 1991b). Malaria transmission is seasonal and reaches a peak in October or November following the rainy season. The main *Plasmodium* species present is *P. falciparum*. Chloroquine resistance was first reported in this region in 1986 (BAYOUMI *et al.*, 1989).

Isolation and characterization of *P. falciparum* clones

The initial isolates were obtained with informed consent from villagers attending a local clinic during the October–November malaria season of 1989. Clones were obtained using the limiting dilution method (ROSARIO, 1981), from a selection of isolates known to exhibit a range of sensitivity to chloroquine and to pyrimethamine. Certain of these isolates were known to be mixed infections by their possession of more than one allele of genes for antigens and other proteins (BABIKER *et al.*, 1991a). Following the cloning procedure, the resulting cultures were shown to be pure clones by ensuring that each haploid clone was monoallelic for each of 2 highly polymorphic antigens (merozoite surface protein [MSP]-1 and MSP-2) when tested in immunofluorescence assays with a panel of allele-specific monoclonal antibodies (CONWAY & MCBRIDE, 1991).

Pulsed field gels

P. falciparum chromosomes were separated by pulsed field gradient gel electrophoresis (PFG) as described previously (BABIKER *et al.*, 1991b), with a basic regime of 22 h, 120 s pulses, 140 V followed by 22 h, 180 s pulses, 140 V, and finally 24 h, 300 s pulses, 120 V. Southern blotting, dot-blotting and hybridization were performed using standard techniques (SAMBROOK *et al.*, 1989).

Measurement of drug sensitivity

Hypoxanthine incorporation assay. Chloroquine-induced inhibition of uptake of [³H]hypoxanthine by the clones was measured using the methods of DESJARDINS *et al.* (1979) and GEARY *et al.* (1983). Tests were carried out on unsynchronized cultures, diluted in RPMI medium containing 10% human serum to 1% parasitaemia and 1% haematocrit, in a final volume of 0.2 mL in microtitre plate wells. [³H]hypoxanthine was added to a final concentration of 5 µCi/mL. Chloroquine sulphate was added to final concentrations ranging from 10 to 160 nM. After 40–44 h incubation, cells were lysed, washed and harvested on to fibre glass filters. Filters were then baked

and counted in a PPO/POPOP*/toluene scintillation fluid supplemented with 30% Triton X-100®. Results were expressed as percentage inhibition compared to the incorporation of label in control wells without drug. The concentration of chloroquine which produced 50% inhibition of parasite growth (IC₅₀) was obtained from the regression line of dose/response curves of parasite inhibition plotted against the logarithm of drug concentration.

Microscopical determinations. Clones were cultured in microtitre plates in serial dilutions of chloroquine, mefloquine and pyrimethamine in complete RPMI medium for 72 h, essentially according to the method of THAI-THONG *et al.* (1983). Each well contained 100 µL, at 1% parasitaemia and 5% haematocrit. After 72 h, thin blood films were made from each well, and the viability assessed by microscopical examination. The results were expressed as the minimum inhibitory concentration (MIC) which killed all, or nearly all, of the parasites.

Effect of verapamil

Drug sensitivity of the parasites to chloroquine, mefloquine and pyrimethamine was assessed with and without the addition of 1 µM verapamil to the cultures. The effect of verapamil alone over a wider range of concentrations (0–5 µM) was also tested on some clones.

Chemicals

[³H]hypoxanthine (40 Ci/mmol) was obtained from Amersham International Ltd, UK; chloroquine sulphate (Nivaquine®) from May and Baker Ltd, UK; pyrimethamine from Wellcome, UK; mefloquine from Hoffman La Roche, Switzerland; and verapamil from Sigma Chemical Company. Glass fibre filters were obtained from Titertek.

of results were obtained.

(i) All the clones from a single isolate possessed chromosomes of identical size, as observed in isolate SUD 111. This suggests that, at the time of cloning, the parasites in these samples were of a clonal type, due either to a clonal-type infection in the patient or to selection of a predominant clone during initial culturing of the uncloned isolate.

(ii) The clones of a single isolate varied in the size of only one or 2 chromosomes. This was most probably due to deletions or accretions in the chromosomes concerned during asexual growth in culture of a clonal-type parasite (WELLEMS *et al.*, 1988). This was seen in isolate SUD 105, in which the 4 clones obtained were karyotypically identical, except that clone 7 had a 300–400 kilobase (kb) deletion of chromosome 10, and clones 9 and 11 both had 200 kb accretions in chromosome 4. These related clones had identical drug sensitivities and possessed the same MSP-1 and MSP-2 alleles.

(iii) Several quite different karyotypes were obtained from a single isolate. This result can be presumed to have been due to the patient's harbouring a mixed infection at the time the parasites were obtained. The clones derived from patients SUD 106 and 124 illustrate this situation. While SUD 106/7 and 106/10 differed from other clones of the same isolate, they were identical to each other, except in the size of 2 chromosomes.

Identical clones have never been isolated from different individuals, a result consistent with the extremely diverse genetic profiles of malaria isolates in general (CREASEY, 1990) and in particular the uncloned Asar isolates from which these clones were derived (BABIKER *et al.*, 1991a, 1991b).

An autoradiograph of a Southern blot of gel 1A probed

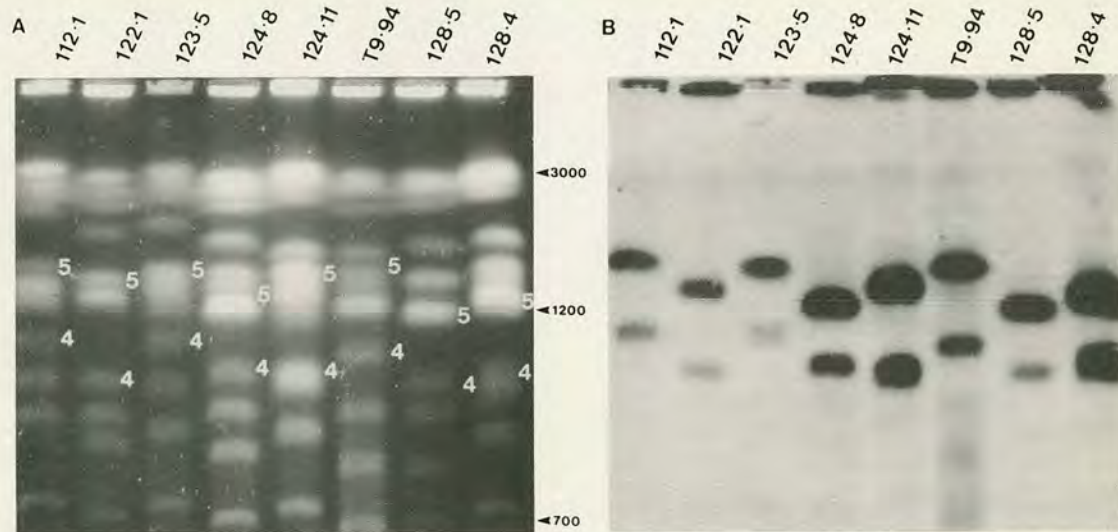


Fig. 1. Karyotypic diversity in some examples of *P. falciparum* clones from Asar village, eastern Sudan. A. Ethidium bromide stained chromosomes separated on a 0.8% agarose gel using a CHEF® electrophoresis apparatus. Seven of the parasites tested are Asar clones, 2 of which, 112/1 and 124/11, were not included in the study (see the Table for the drug sensitivity phenotypes). T9/94 is a Thai clone. Chromosomes 4 and 5 are marked, and approximate chromosome sizes in kilobases are indicated on the right. B. After Southern blotting, gel A was successively probed with gene markers for chromosomes 4 and 5, the *Pgh1* gene and the *DHFR* gene respectively. The 2 autoradiographs from the same blot have been superimposed before photography for comparison and signal intensities are not proportional to gene copy number.

Results

Clone characterization

All the clones were confirmed as pure by their possession of single alleles of MSP-1 and MSP-2, using monoclonal antibody typing (results not shown).

The clones were examined for chromosomes by pulsed field electrophoresis. Results for a representative sample of clones are shown in Fig. 1A. When clones from individual isolates were compared, the following 3 categories

with the genes encoding the *P. falciparum* dihydrofolate reductase (*DHFR* gene) located on chromosome 4, and *Pgh1* located on chromosome 5 is shown in Fig. 1B. In each clone the *DHFR* probe always hybridized to the fourth smallest chromosome, which, however, differed in size among the clones. The *Pgh1* probe hybridized to the fifth smallest chromosome in only 2 clones (SUD 128/5 and SUD 128/4). These results illustrate clearly the considerable size polymorphism of these 2 chromosomes, especially of chromosome 5, among this group of isolates.

*2,5-Diphenyloxazole/1,4-bis[5-phenyl-2-oxazolyl]benzene.

Although there were differences in the relative intensity of hybridization of the probes shown in Fig. 1, deoxyribonucleic acid (DNA) transfer from pulsed field gels is not reliably quantitative and the amount of DNA per lane in this gel was not constant. Using more quantitative dot-blots and Southern blots of DNA digested with restriction enzymes, we have not been able to detect *DHFR* or *Pgh1* gene amplification in DNA samples of any of the clones, whether drug-resistant or drug-sensitive.

Chloroquine sensitivity

Drug resistance measurements, even with identical clones, may show considerable inter-laboratory variation. To increase confidence in assessing the drug response of a given clone, we have, therefore, employed and compared 2 different assays in this work. IC₅₀ values above 35 nM, as assessed by [³H]hypoxanthine incorporation, and MIC values of 16 × 10⁻⁷ M, as assessed microscopically, were taken to indicate clear chloroquine resistance. The drug responses of the 20 clones are presented in the Table. They are listed in ascending order of IC₅₀ values,

Table. Drug sensitivity phenotypes of twenty *P. falciparum* clones derived from patient blood samples collected in the village of Asar, Sudan, during October and November 1989

Verapamil	Chloroquine ^a IC ₅₀ ^b (× 10 ⁻⁹ M)		Chloroquine ^a MIC ^c (× 10 ⁻⁷ M)		Mefloquine ^a MIC ^c (× 10 ⁻⁷ M)		Pyrimethamine ^a MIC ^c (M)	
	No	Yes	No	Yes	No	Yes	No	Yes
Experimental clones ^d								
SUD 105/1	4	3	8	4	4	4	10 ⁻⁷	10 ^{-7f}
SUD 105/9	6	5	8	4	4	4	10 ⁻⁷	10 ^{-7f}
SUD 105/11	7	4	8	4	4	4	10 ⁻⁷	10 ^{-7f}
SUD 106/10	7	4	2	2	4	4	10 ^{-6c}	10 ⁻⁶
SUD 105/7	8	4	8	4	4	4	10 ⁻⁷	10 ^{-7f}
SUD 106/9	9	8	4	4	4	4	10 ⁻⁶	10 ⁻⁶
SUD 106/7	13	4	4	2	4	4	10 ^{-6c}	10 ⁻⁶
SUD 106/11	11	5	4	4	4	4	10 ⁻⁶	10 ⁻⁶
SUD 106/1	15	6	2	2	4	4	10 ^{-6c}	10 ⁻⁶
SUD 128/5	43	10	8	4	4	2	10 ⁻⁸	10 ^{-8f}
SUD 128/4	45	11	8	4	4	2	10 ⁻⁸	10 ^{-8f}
SUD 124/8	58	19	16 ^e	4	2	2	10 ⁻⁵	10 ^{-5f}
SUD 128/1	64	18	8	4	4	2	10 ⁻⁸	10 ⁻⁹
SUD 123/5	64	18	16	8	8	8 ^f	10 ^{-5c}	10 ⁻⁵
SUD 124/5	65	28	16	4	2	2	10 ⁻⁵	10 ⁻⁵
SUD 124/1	73	27	16	8	2	2	10 ⁻⁵	10 ^{-6f}
SUD 126/1	83	18	16 ^f	1	2	2	10 ⁻⁷	10 ⁻⁷
SUD 102/1	85	12	16	8	2	2	10 ⁻⁸	10 ⁻⁹
SUD 122/1	92	23	16	4	2	2	10 ⁻⁶	10 ^{-6f}
SUD 111/1	95	21	16	8	2	2	10 ^{-5c}	10 ⁻⁵
Control clones								
3D7 ^g	8	3	4	2	4	2	10 ⁻⁸	10 ⁻⁹
Dd2 ^g	57	15	16	8	16	8	10 ⁻⁶	10 ^{-6f}

^aAll values are means of 3 or more separate experiments, with ('yes') and without ('no') verapamil (1 μM).

^bConcentration giving 50% inhibition of parasite growth.

^cConcentration killing all parasites within 72 h.

^dClones with the same 'one hundred' number before the solidus were isolated from a single patient sample.

^eA few parasites were still viable at the indicated concentrations.

^fReduced parasitaemia at the indicated concentrations.

^g'Control' chloroquine-sensitive and chloroquine-resistant clones, respectively.

with the chloroquine-resistant Dd2 and chloroquine-sensitive 3D7 laboratory-adapted clones as reference controls.

On the whole, the 2 tests of sensitivity *in vitro* to chloroquine gave comparable results. No clone with an MIC of 16 × 10⁻⁷ M chloroquine had an IC₅₀ < 50 nM. Similarly, no clone with an IC₅₀ value < 35 nM has an MIC > 8 × 10⁻⁷ M. Certain ambiguities remain, in that some clones of isolate SUD 105 which appeared very chloroquine-sensitive in the IC₅₀ test, showed intermediate sensitivity in the MIC test. Apart from this, the combination of both tests appeared to allow differentiation of the parasites into a sensitive group comprising all the SUD 105

and SUD 106 clones, and a resistant group consisting of all the other clones. No clone had chloroquine sensitivity in the IC₅₀ range 15–43 nM.

Resistance to mefloquine and pyrimethamine

Most of the clones were sensitive to mefloquine (Table), in agreement with the results of tests on the original 29 uncloned isolates from Asar village (BABIKER *et al.*, 1991b). Again in accordance with our earlier results, 5 clones (SUD 124/1, SUD 124/5, SUD 124/8, SUD 123/5 and SUD 111/1) were highly resistant to pyrimethamine.

One clone, SUD 123/5, was resistant to both chloroquine and pyrimethamine and showed a slight decrease in susceptibility to mefloquine. However, none of the other clones was resistant to all 3 drugs. Mefloquine resistance is clearly not linked to chloroquine resistance. While high level pyrimethamine resistance was found only in chloroquine-resistant clones, many of the latter were sensitive to pyrimethamine.

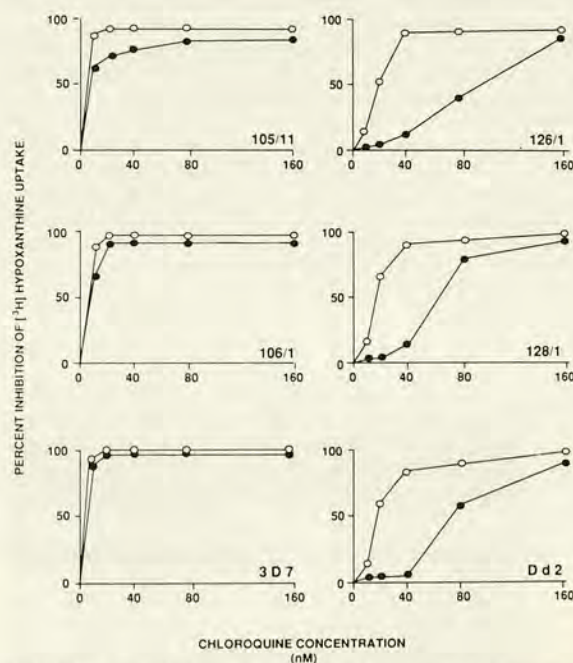


Fig. 2. The effect of 1 μM verapamil on the response of 4 Sudanese and 2 standard laboratory-adapted *P. falciparum* clones to chloroquine. Closed circles (●), without verapamil; open circles (○), with verapamil. SUD 105/11, SUD 106/1 and 3D7 were chloroquine sensitive, SUD 126/1, SUD 128/1 and Dd2 were chloroquine resistant. Points represent the average of simultaneous duplicate experiments. Similar curves have been obtained for all clones tested.

Effect of verapamil

Verapamil, a calcium channel blocker, has been reported to reverse chloroquine resistance in some isolates of *P. falciparum* (MARTIN *et al.*, 1987). We tested the effects of verapamil on our clones (Table) to ascertain whether verapamil reversibility was also characteristic of the chloroquine-resistant parasites studied here. Examples of drug tests where the capacity of increasing concentrations of chloroquine to inhibit [³H]hypoxanthine uptake has been measured, with and without the addition of 1 μM verapamil, are shown in Fig. 2. Clones SUD 105/11, SUD 106/1 and 3D7 were chloroquine-sensitive. Verapamil did not shift the inhibition curve of the 3D7 reference control, although it slightly increased the toxicity of chloroquine to the drug-sensitive Asar (SUD) clones. Clones SUD 126/1, SUD 128/1 and Dd2 were chloroquine-resistant, and verapamil clearly increased their chloroquine sensitivity, although not to the levels of the naturally sensitive clones.

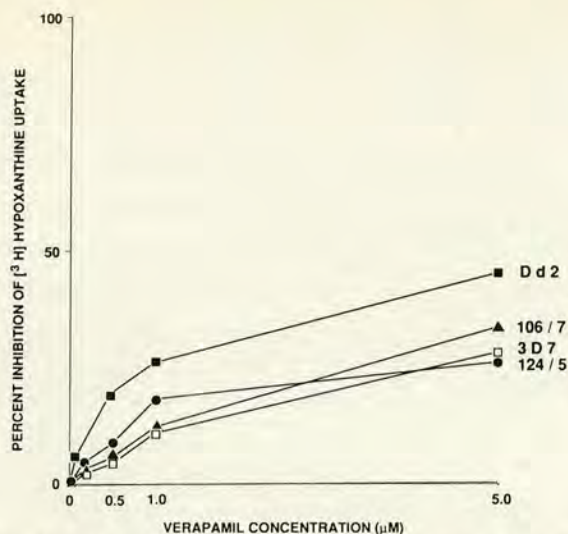


Fig. 3. The effect of verapamil alone on [3 H]hypoxanthine uptake over 40–44 h by 2 Sudanese and 2 standard laboratory-adapted *P. falciparum* clones. Dd2 and SUD 124/5 were chloroquine resistant and 3D7 and SUD 106/7 were chloroquine sensitive. Points represent the average of duplicate experiments carried out simultaneously on the same microtitre plate. Three separate experiments gave the same rank order of drug sensitivities.

It has been reported (MARTIN *et al.*, 1987) that verapamil itself has intrinsic antimalarial activity. An example of the effect of verapamil alone on the capacity of parasites to incorporate [3 H]hypoxanthine is shown in Fig. 3. Dd2 and SUD 124/5 were chloroquine-resistant, and SUD 106/7 and 3D7 were chloroquine-sensitive. There were large intrinsic differences in their susceptibility to the antimalarial effect of verapamil, Dd2 being particularly sensitive.

The sensitivity of some of the Sudanese clones to mefloquine and pyrimethamine also appeared to increase on addition of verapamil (Table), although to only a limited extent.

Discussion

In our initial survey of uncloned *P. falciparum* isolates from Asar village, we found that no 2 isolates were genetically identical and that it was possible to detect several obviously mixed isolates (BABIKER *et al.*, 1991a, 1991b). The molecular karyotypes of the clones derived from these isolates have further confirmed the highly diverse nature of this small parasite population. Mapping of *DHFR* and *Pgh1* markers on to chromosomes demonstrates that comparisons of chromosome separations stained with ethidium bromide underestimate the true extent of karyotypic diversity. More detailed genome maps would undoubtedly reveal more radical differences in genome organization between these clones which, it should be emphasized, represent only a small sample of the total population of *P. falciparum* in this small community.

In general, our results indicated that the biochemical characteristics of the Sudanese chloroquine-resistant *P. falciparum* are similar to those reported for south-east Asian and Latin American isolates, and are consistent with there being a similar molecular basis for the phenomenon. The use of genetically pure clones from the 1989 transmission peak permitted a clearer differentiation of some of the characteristics of the drug resistant *P. falciparum* in this village, as follows.

(i) Clones of *P. falciparum* exhibiting low, intermediate and high level chloroquine resistance co-existed with highly sensitive parasites in the population of this village.

(ii) The reversibility of chloroquine resistance of *P. falciparum* by verapamil (MARTIN *et al.*, 1987) also appeared to be characteristic of the chloroquine resistant malaria currently spreading in Asar. However, verapamil has an antimalarial effect of its own, as shown by MARTIN *et al.* (1987) and in our work. The addition of verapamil increased the sensitivity of these clones not only to chloroquine but also, in some instances to mefloquine and pyrimethamine.

(iii) The *Pgh1* gene has been reported to be amplified in some, but not all, chloroquine-resistant *P. falciparum* isolates (FOOTE *et al.*, 1989). However, we have not been able to detect *Pgh1* gene amplification in DNA samples from either chloroquine-resistant or chloroquine-sensitive Sudanese *P. falciparum* clones.

(iv) In accordance with our earlier findings with the uncloned isolates, 5 clones were highly resistant to pyrimethamine. This result is of interest, since pyrimethamine was not widely used in Sudan before 1986. Since then, however, Fansidar® (pyrimethamine/sulfadoxine) became available through relief agencies. This may explain the appearance and selection of mutants resistant to this drug. It may also be relevant that clinical pyrimethamine resistance was noted in Sudan as early as 1954 (PHILLIPS, 1954) and, more recently, in the Sennar region (IBRAHIM *et al.*, 1991). Only a few parasites in this and our previous survey (BABIKER *et al.*, 1991b) showed any degree of resistance to mefloquine.

(v) In the survey of the uncloned isolates, some were found to exhibit resistance to all 3 drugs. However, none of the clones examined in this work was multi-drug resistant, with the possible exception of clone SUD 123/5. Since clones resistant to both chloroquine and, at a high level, to pyrimethamine coexisted in this community, and many patients were infected with more than one clone, it is certainly possible that genetic recombination during mosquito transmission could increase the frequency of multi-drug resistant clones.

These results suggest that chloroquine-resistant *P. falciparum* in this small village (and presumably the rest of Sudan) is not a distinct parasite 'strain'. Rather, it appears that genes conferring resistance to this drug are increasing in frequency in the parasite population, probably due to the continuing use of chloroquine in the area. While clinical chloroquine resistance has increased in frequency among patients attending the nearby Gadaref District Hospital during 1986–1990 (unpublished data), most patients in the village appeared to be cured by standard chloroquine therapy. Whether the situation is slowly deteriorating and chloroquine will soon be clinically useless remains to be seen.

Acknowledgements

We thank Drs Thor Theander and Lars Hviid of Copenhagen State University Hospital for their help and support in the Sudan Malaria Project. We also thank the Malaria Administration of the Sudanese Ministry of Health for their advice and support for this project. Dr Jana McBride kindly supplied us with her panel of *P. falciparum* allele-specific monoclonal antibodies, and Drs John Hyde and Alan Cowman kindly supplied probes for the *DHFR* and *Pgh1* genes. This work was supported by the Wellcome trust, the UK Medical Research Council, US NIH grant A1-16312 and WHO (TDR) Institution Strengthening Grant 890297. Hamza Babiker and Ali Sultan are supported by British Council Studentships, Riad Bayoumi and David Arnot held a Wellcome Travelling Fellowship and Senior Biomedical Research Fellowship, respectively. This work would not have been possible without the support and goodwill of the staff of Gedaref Hospital and the villagers of Asar, Sudan.

References

- Al Tawil, N. & Akood, M. A. (1983). *Response of falciparum malaria to a standard regimen of chloroquine in Khartoum Province, Sudan*. Geneva: World Health Organization, mimeographed document no. WHO/MAL/83.991.
- Babiker, H. A., Creasey, A. M., Fenton, B., Bayoumi, R. A., Arnot, D. E. & Walliker, D. (1991a). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens. *Transactions*

- of the Royal Society of Tropical Medicine and Hygiene, 85, 572-577.
- Babiker, H. A., Creasey, A. M., Bayoumi, R. A., Walliker, D. & Arnot, D. E. (1991b). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the *mdr1* genotype of recent isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85, 578-583.
- Bayoumi, R. A., Babiker, H. A., Ibrahim, S. M., Galib, H. W., Saeed, B. O., Khider, S. & Karim, E. A. (1989). Spread of chloroquine resistant *Plasmodium falciparum* in Sudan. *Acta Tropica*, 46, 157-165.
- Conway, D. J. & McBride, J. S. (1991). Population genetics of *Plasmodium falciparum* within a malaria hyperendemic area. *Parasitology*, 103, 7-16.
- Creasey, A., Fenton, B., Walker, A., Thaithong, S., Oliveria, S., Mutambu, S. & Walliker, D. (1990). Genetic diversity of *Plasmodium falciparum* shows geographical variation. *American Journal of Tropical Medicine and Hygiene*, 42, 403-413.
- Desjardins, R. E., Canfield, C. J., Hyman, J. D. & Chulay, J. D. (1979). Quantitative assessment of antimalarial activity *in vitro* by a semiautomated technique. *Antimicrobial Agents and Chemotherapy*, 16, 710-718.
- Foot, S. J., Thompson, J. K., Cowman, A. F. & Kemp, D. J. (1989). Amplification of the multidrug resistance gene in some chloroquine resistant isolates of *Plasmodium falciparum*. *Cell*, 57, 921-930.
- Foot, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M., Forsyth, K., Kemp, D. J. & Cowman, A. F. (1990). Several alleles of the multidrug resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature*, 345, 255-258.
- Geary, T. G., Divo, A. A. & Jensen, J. B. (1983). An *in vitro* assay system for the identification of potential antimalarial drugs. *Journal of Parasitology*, 69, 577-583.
- Ginsberg, H. (1988). Effect of calcium antagonists on malaria susceptibility to chloroquine. *Parasitology Today*, 4, 209-211.
- Homewood, C. A., Warhurst, D. C., Peters, W. & Babbaley, V. C. (1972). Lysosomes, pH and the antimalarial action of chloroquine. *Nature*, 235, 50-52.
- Ibrahim, M. E., Awad-El-Kariem, E. M., El Hassan, I. M. & El Mubarak, E. R. M. (1991). A case of *Plasmodium falciparum* malaria sensitive to chloroquine but resistant to pyrimethamine/sulphadoxine in Sennar, Sudan. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85, 446.
- Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. M. J., Martin, S. K., Milhous, W. K. & Schlesinger, P. H. (1987). Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science*, 238, 1283-1285.
- Martin, S. K., Oduola, A. M. J. & Milhous, W. K. (1987). Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science*, 235, 899-901.
- Meshnik, S. (1990). Chloroquine as intercalator: a hypothesis revived. *Parasitology Today*, 6, 77-79.
- Peters, W. (1987). *Chemotherapy and Drug Resistance in Malaria*. London: Academic Press.
- Phillips, M. G. (1954). Malaria prophylaxis. *British Medical Journal*, i, 155.
- Rosario, V. (1981). Cloning of naturally occurring mixed infections of malaria parasites. *Science*, 212, 1037-1038.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Thaithong, S., Beale, G. H. & Chutmongkonkul, M. (1983). Susceptibility of *Plasmodium falciparum* to five drugs: an *in vitro* study of isolates mainly from Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 77, 228-231.
- Warhurst, D. C. (1988). Mechanism of chloroquine resistance in malaria. *Parasitology Today*, 4, 211-213.
- Wellems, T. E., Oduola, A. M. J., Fenton, B., Desjardins, R., Panton, L. J. & Do Rosario, V. E. (1988). Chromosome size variation occurs in cloned *Plasmodium falciparum* on *in vitro* cultivation. *Revista Brasileira de Genetica*, 11, 813-825.
- Wellems, T. E., Panton, L. J., Gluzman, I. Y., Rosario, V. E., Gwadz, R. W., Walker-Jonah, A. & Krogstad, D. J. (1990). Chloroquine resistance not linked to *mdr*-like genes in *Plasmodium falciparum* cross. *Nature*, 345, 253-255.

Received 26 August 1992; revised 19 October 1992; accepted for publication 21 October 1992

Announcements

The Tropical Health and Education Trust

Fellows of the Society have always been actively involved in many tropical countries in establishing and developing medical schools and other training institutions. But some of these schools, particularly in poorer African countries, face severe hardships. Students have no books, there is no foreign exchange for journals, equipment lacks spares, research cannot be supported and external aid is directed towards primary health care.

The Tropical Health Education Trust has started to relieve, with support from many individuals, Trusts and organizations, some of these disadvantages.

Basic books have been sent to all the rural hospitals in two African countries, sets of books have been given for students in a number of others. Links between medical schools overseas and home departments have been started with fellowships for students in training and research methods also.

The Tropical Health and Education Trust aims to extend support like this to more countries, hospitals, medical schools and students and needs funds to do it: Fellows of the Society who would like to take this opportunity to help our colleagues overcome some of their obstacles can do so through a single gift, a four-year or a deposited covenant, or even through a legacy.

Trustees include: R. M. Anderson, K. P. W. J. McAdam, E. H. O. Parry (Chairman), D. A. Warrell.

For more information about THET please write or telephone: 21 Edenhurst Avenue, Fulham, London, SW6 3PD, UK. Tel: 071 927 2411, Fax: 071 637 4314.

Eighth International Conference on Trichinellosis

7-11 September 1993

Palazzo dei Congressi, Orvieto, Italy

Further information from: Dr Edoardo Pozio, Laboratory of Parasitology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy; telephone +39 6 4990 (extension 304), fax +39 6 4469823, telex 610071 ISTISAN.

**The follow pages were poor originals from the
HardCopy Theses**

Genetic recombination in field populations of *Plasmodium falciparum*

D. Walliker, L.C. Ranford-Cartwright and H.A. Babiker

Malaria parasites undergo a mainly haploid life-cycle. The only diploid stage is the zygote, formed by fusion of gametes in the mosquito stomach. The first division of the zygote is a meiotic one, producing, after further mitotic divisions, haploid sporozoites. Genetic recombination occurs at meiosis, following cross-fertilization of gametes of parasites with different genotypes. This has been shown in laboratory studies by feeding mosquitoes on a mixture of *Plasmodium falciparum* clones and analyzing the resulting progeny for parasites with non-parental combinations of the clone markers. Such recombinants are produced at a higher than expected frequency.

There is considerable genotype diversity in field populations of *P. falciparum*. Evidence that recombination in mosquitoes is the principal cause of this diversity is two-fold. First, parasites isolated from patients in small isolated communities at the same time are genetically very diverse. No two isolates examined for polymorphic markers at some 20 loci have been found to possess identical combinations of the allelic variants of these genes. Second, examination of oocysts in wild-caught mosquitoes by the PCR technique has shown that a high proportion are heterozygotes. There is thus frequent crossing in natural populations of this parasite.

In addition to recombination at meiosis, it is also clear that genetic changes can occur during asexual multiplication of *P. falciparum* blood forms, as shown by deletions of regions of certain chromosomes during *in vitro* culture. The extent to which this occurs in nature is not known.

INTRODUCTION

Genetic recombination is classically defined as the process by which organisms with novel combinations of genes are produced in crosses between two parent organisms. Recombination occurs primarily at meiosis, in two principal ways: (i) independent segregation of variant forms (alleles) of genes on different chromosomes and (ii) crossing-over events between linked genes on the same chromosome. A third aspect that must be considered is intragenic recombination, by which novel alleles of a given gene can be formed by crossing-over events within the gene itself. This process is likely to occur very rarely, but may be significant where selection pressures against existing alleles are high. Recombination at mitosis also occurs, although much more rarely. In the case of haploid organisms such as *Plasmodium*, mitotic recombination is not expected to have important genetic consequences, since it can only involve exchange between identical (sister) chromatids, which contain identical alleles of any given gene.

It is now clear that recombination is of primary importance in generating the genetic diversity seen in natural populations of *Plasmodium falciparum*. We review here briefly current knowledge on the organization of the parasite genome and on ways in which chromosome diversity arises. Data on recombination in this parasite both in laboratory and field studies are then discussed.

ORGANIZATION OF THE *PLASMODIUM FALCIPARUM* GENOME

The nuclear genome of *P. falciparum*, and most probably of other species of *Plasmodium*, consists of 14 chromosomes. They range in size from 0.65 to 3.4 megabases (Mb) (Triglia *et al.*, 1992), and are visible only by pulsed-field gradient gel electrophoresis (PFGE). Two cytoplasmic genetic elements are also present, a linear element of tandemly repeated units of 6 kb which contains mitochondrial genes and a 35 kb circular DNA element which encodes chloroplast-like genes (Wilson *et al.*, 1991).

Genes can be mapped to chromosomes by hybridization of gene probes to blots of pulsed-field gels (Triglia *et al.*, 1992). The linear order of genes is also being elucidated by this approach, using blots of chromosome fragments cut by rare-cutting restriction enzymes (Sinnis and Wellems, 1988; Corcoran *et al.*, 1988) and of yeast artificial chromosome (YAC) constructs from certain chromosomes (Triglia and Kemp, 1991).

GENETIC EVENTS IN THE LIFE-CYCLE

The parasite is haploid for most of its life-cycle. The only diploid form is the zygote, formed by union of gametes in the mosquito midgut. Meiosis occurs within a few hours of zygote formation (Sinden and Hartley, 1985), resulting eventually in the production of haploid sporozoites in each oocyst.

Recombination during meiosis is expected to be responsible for the formation of most novel genotypes. Laboratory crossing work has demonstrated that this process is very efficient in *Plasmodium*. In these studies, deliberate mixtures of parasite clones are fed to mosquitoes in order to allow crossing between gametes of each clone. The resulting sporozoites are used to establish new infections, and the resultant organisms examined for the presence of parasites with non-parental (recombinant) combinations of characters. Numerous crosses done with rodent malaria species and with *P. falciparum* have shown that recombinants are produced in this way at a high frequency (Walliker, 1989).

A recent advance in genetic analysis of malaria parasites is the ability to examine the genetic composition of oocysts in mosquitoes. This has been achieved by using the polymerase chain reaction (PCR) on certain *P. falciparum* antigen genes (Ranford-Cartwright *et al.*, 1991). When oocysts derived from mosquitoes fed on a mixture of two clones, denoted 3D7 and HB3, were examined in this way, approximately 50% were found to be heterozygous for the parent genes (Ranford-Cartwright *et al.*, 1993). This showed directly that random fertilization events had occurred between the gametes of each clone, producing hybrid and parental type zygotes. In the hybrid zygotes, meiotic recombination leads to the production of haploid recombinant forms. The inheritance of the 6 kb cytoplasmic element has recently been studied. In crosses between *P. falciparum* clones, oocysts found to be hybrid for nuclear gene markers proved to have only a single parental form of this element (Creasey *et al.*, 1993). Such uniparental inheritance is typical for mitochondria in many other organisms.

CHROMOSOME POLYMORPHISM

A striking early finding on *Plasmodium* chromosomes was the remarkable variation in the size of homologous chromosomes in different isolates and clones. This could be seen in

parasites taken directly from the blood (Triglia *et al.*, 1991b), and seen in the past five years, it is clear that these size polymorphisms

Crossing-over at

As might be expected, recombination events. This has been demonstrated with different size chromosomes which possessed a crossover in the chromosome interval. Extensive crossing-over was observed in a cross by examining the products (Walker-Jonah *et al.*

Chromosome Breakage

There are several examples of *P. falciparum* maintaining a stable karyotype. For example, Scherf *et al.* (1991) found that a gametocyte-specific cloned parasite line maintained this clone. Similar results were obtained with (KAHRP) on chromosomes. The breakage point is not understood. It seems that some sequences are not protected (Mattei, 1992).

Increases in Chromosome

There are occasional reports of prolonged asexual phases in *P. berghei* (Janse *et al.*, 1991). The additions of a 2.3 kb element to some of this species, and a line of *P. berghei* with a translocated portion of the genome, occurred during asexual

parasites taken directly from different infected patients (Corcoran *et al.*, 1986; Babiker *et al.*, 1991b), and so the phenomenon was clearly not an artefact of *in vitro* culture. In the past five years, it has become clear that several mechanisms are involved in generating these size polymorphisms, as follows:

Crossing-over at Meiosis

As might be expected for a eukaryotic organism, meiosis results in frequent crossing-over events. This has been demonstrated in laboratory crosses between *P. falciparum* clones with different sized chromosomes. For example, in a cross between clones 3D7 and HB3 which possessed a different-sized chromosome 4, Sinnis and Wellems (1988) showed that a crossover in the central region of this chromosome accounted for the production of a chromosome intermediate in size between those of the parent clones used in the cross. Extensive crossing-over events in all 14 chromosomes have been demonstrated in another cross by examining the inheritance patterns of numerous chromosome-specific markers (Walker-Jonah *et al.*, 1992).

Chromosome Breakage During Asexual Division

There are several reports of deletions of portions of chromosomes in blood forms of *P. falciparum* maintained in culture. This happens especially near chromosome ends. For example, Scherf *et al.* (1992) showed that a large part of a sub-telomerically located gametocyte-specific gene (Pf11.1) on chromosome 10 became deleted during culture of a cloned parasite line. Parasites with the truncated gene were found among sub-clones of this clone. Similar findings have been made with a knob-associated histidine-rich protein (KAHRP) on chromosome 2 (Pologe and Ravetch, 1988). New telomeres are formed at the breakage points in these instances. The mechanisms by which this occurs are not understood. It seems unlikely that recombination is involved since telomeric repeat sequences are not present at any of the breakpoints which have been studied (Scherf and Mattei, 1992).

Increases in Chromosome Size

There are occasional reports of an increase in the size of certain chromosomes following prolonged asexual passage. This has been studied particularly in the rodent malaria species *P. berghei* (Janse *et al.*, 1989). In some instances, these increases appear to be due to additions of a 2.3 kb sequence commonly found in subtelomeric sites on several chromosomes of this species (Dore *et al.*, 1990; Pace *et al.*, 1990). Janse *et al.* (1992) describes a line of *P. berghei* in which a chromosome of larger than normal size appeared to contain a translocated portion of another chromosome, although it was not clear whether this had occurred during asexual passage of this parasite.

Gene Amplification

Amplification of genes in *Plasmodium* has been studied particularly with respect to the so-called multi-drug resistance (mdr) genes, which have been thought to be involved in resistance to chloroquine. Foote *et al.* (1989) described lines and clones of *P. falciparum* with differing numbers of copies of the Pfmdr1 gene, arranged in tandem arrays on chromosome 5, which, they considered, were responsible for the size polymorphisms seen in this chromosome. There was some evidence of instability of such tandem arrays in one clone (B8) during culture.

In nature, it can be expected that meiotic recombination is the principal mechanism for the generation of chromosomal polymorphisms. While the other phenomena outlined above have been shown to occur frequently in laboratory cultures, the extent to which they occur in nature is not known.

RECOMBINATION IN NATURAL PARASITE POPULATIONS

The extent of crossing, and hence of recombination, in nature is now being addressed. The topic has been the subject of some recent debate (Tibayrenc and Ayala, 1991; Walliker, 1991; Dye, 1991). If recombination occurs rarely, or never at all, one would expect to find in circulation only a limited number of genetically different parasites, with some forms being over-represented. If it is frequent, then a large diversity of parasite clones would be expected, exhibiting all possible combinations of the alleles of polymorphic genes.

This subject can be examined in two ways. First, the genotypes of parasites in patients can be examined to determine whether their frequencies are in accordance with a randomly interbreeding model. Second, direct evidence of crossing can be sought by determining the frequency of hybrid oocysts in wild-caught mosquitoes.

Diversity of Parasites in the Blood of Patients

Characters Studied

As mentioned above, blood forms of *P. falciparum* from different patients can be examined for gross genetic differences by comparing their chromosomes, using the PFGE technique. More precise genetic differences can be studied by examining allelic variation in specific genes. Characters available for such work include enzymes and other proteins revealed by electrophoretic techniques, antigens using monoclonal antibodies and variations in drug-sensitivity (Creasey *et al.*, 1990). Each of these characters requires the availability of cultured parasite material.

In recent years, the polymerase chain reaction (PCR) technique has been developed to amplify alleles of genes whose DNA sequence is known. Two merozoite surface antigens, MSP-1 and MSP-2, have proved particularly useful in this regard (Snewin *et al.*, 1991). Each contains regions of repetitive sequences, and alleles of each gene vary in both the numbers of repeats present and in their sequence. The PCR-amplified products of these regions can be examined for size variation by electrophoresis, and by blotting and

hybridization with probes. One of the advantages of this technique is that it can be used for example in field studies.

Results

Numerous studies have been carried out on other species in the genus *Plasmodium* and on the frequency of recombination in *P. falciparum* and *P. falciparum* antigens in *P. falciparum* (McBride, 1991).

(i) There is considerable diversity of antigens. For example, the antigen MSP-1, a major antigen of *P. falciparum*, is found in all isolates of *P. falciparum* (Ranford-Cartwright, 1991).

The same gene among different isolates has come from different sources (al. (1987), although

(ii) No two isolates are identical. This is the case even in the same patient (Babiker *et al.*, 1991). It is expected to be in accordance with a randomly interbreeding model.

(iii) Mixed infections are common.

(iv) There is genetic diversity. For example, the ADA-2 is very rare.

Diversity of Parasites in the Blood of Patients

Work is now starting to be done on mosquitoes in villages in the Charwood and Freetown areas, endemic and semi-endemic. Mixed infections are common. Mixed infections are examined in *Anopheles* and *Culiseta* proved to be heterogeneous.

CONCLUSION

The genetic diversity of *Plasmodium* is considerable and is being studied.

hybridization with allele-specific oligonucleotides for sequence differences. A particular advantage of this technique is that it can be performed on small quantities of parasite material, for example in fingerprick blood samples, without the need for culturing (Foley *et al.*, 1992).

Results

Numerous studies have now been carried out on genetic diversity in *P. falciparum* and other species in various countries. Those most relevant to answering questions on the frequency of recombination include those on enzymes in *P. chabaudi* (Beale *et al.*, 1978) and *P. falciparum* (Carter and Voller, 1975), and on enzymes, 2D-PAGE proteins and antigens in *P. falciparum* (Creasey *et al.*, 1990; Babiker *et al.*, 1991a, 1991b; Conway and McBride, 1991). The principal findings from all these studies are:

(i) There is considerable allelic diversity of many genes, especially those encoding antigens. For example, in *P. falciparum* Conway and McBride (1991) found 36 alleles of antigen MSP-1, differing by epitopes recognized by monoclonal antibodies, among isolates of *P. falciparum* from a peri-urban region in the Gambia. H.A. Babiker and L.C. Ranford-Cartwright (unpublished data) have used PCR to differentiate 22 alleles of the same gene among only 50 isolates in Tanzania. It is quite probable that this diversity of alleles has come about by extensive intragenic recombination, as suggested by Tanabe *et al.* (1987), although formal proof for this has not been obtained in crossing experiments.

(ii) No two isolates have been found in which parasites possess identical genotypes. This is the case even in a small community in which malaria transmission is highly seasonal (Babiker *et al.*, 1991a, 1991b) and in which a restricted number of genotypes might be expected to be in circulation.

(iii) Mixed infections with more than one genetically distinct clone are common.

(iv) There is geographical variation in the frequencies with which alleles of many genes occur. For example, the frequency of an electrophoretic form of adenosine deaminase denoted ADA-2 is very rare in Thailand and the Gambia, but common in Brazil (Creasey *et al.*, 1990).

Diversity of Parasites in Mosquitoes

Work is now starting to examine the genotypes of oocysts of *P. falciparum* in wild-caught mosquitoes in villages near Ifakara, Tanzania (H.A. Babiker, L.C. Ranford-Cartwright, D. Charlwood and P. Billingsley, unpublished data). This is a region where malaria is highly endemic and some 60% of the inhabitants exhibit *P. falciparum* parasitaemias at any given time. Mixed infections with more than one clone are common. Sixty-eight percent of oocysts examined in *Anopheles gambiae* and *A. funestus*, caught in houses of these villages, have proved to be heterozygous for alleles of MSP-1, MSP-2, or of both genes.

CONCLUSIONS

The genetic diversity seen in natural infections of *P. falciparum* has now been shown to be considerable. As discussed above, chromosomal polymorphisms are extensive in

different parasite isolates, even in those from small communities (Babiker *et al.*, 1991b). There is also remarkable allelic variation of many individual genes, especially those encoding antigens. Perhaps the most significant finding is that no two isolates yet studied have identical genotypes.

It is now quite clear that the numerous combinations of alleles of the genes studied seen among clones in natural infections are evidence of extensive recombination in nature. This presumably happens as a consequence of crossing between clones when mosquitoes take up mixtures of gametocytes. Self-fertilization events between genetically identical gametes also occur in such instances. In addition, selfing is the only possibility if mosquitoes take up gametocytes of a single clone. In Tanzania, some 32% of oocysts are homozygous at two highly polymorphic loci, and these are almost certainly derived from such selfing events.

Thus, evidence from population studies of *P. falciparum* strongly supports the view that gametes of this parasite undergo random mating. When cross-mating occurs, recombination along classical Mendelian lines ensures that recombinants are produced readily. The 'clonality' model proposed by Tibayrenc *et al.* (1990) is thus inappropriate when applied to this parasite. It could, at least in theory, apply in regions where clonal infections are the norm, in which mosquito transmission would maintain pure clones. In practice, no regions with infections of this type have yet been found.

ACKNOWLEDGEMENTS

We are happy to acknowledge support from the Medical Research Council of Great Britain, the World Health Organization, and the Commission of the European Communities for some of the work described here.

REFERENCES

- BABIKER, H.A., CREASEY, A.M., FENTON, B., BAYOUMI, R.A.L., ARNOT, D.E. and WALLIKER, D. 1991a. Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 85: 572-577.
- BABIKER, H.A., CREASEY, A.M., BAYOUMI, R.A.L., WALLIKER, D. and ARNOT, D.E. 1991b. Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the *mdr1* genotype of recent isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 85: 578-583.
- BEALE, G.H., CARTER, R. and WALLIKER, D. 1978. Genetics. In: Killick-Kendrick, R. and Peters, W., eds. *Rodent Malaria*. London: Academic Press, pp. 213-245.
- CARTER, R. and VOLLER, A. 1975. The distribution of enzyme variation in populations of *Plasmodium falciparum* in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 69: 371-376.
- CONWAY, D.J. and McBRIDE, J.S. 1991. Population genetics of *Plasmodium falciparum* within a malaria hyperendemic area. *Parasitology* 103: 7-16.
- CORCORAN, L.M., FORSYTH, K.P., BIANCO, A.E., BROWN, G.V. and KEMP, D.J. 1986. Chromosome size polymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite populations. *Cell* 44: 87-95.

- CORCORAN, L.M., bination within *Plasmodium falciparum*.
- CREASEY, A., FENTON, B., WALLIKER, D. 1991. *American Journal of Tropical Medicine and Hygiene* 44: 100-105.
- CREASEY, A.M., RAMESH, R.J.M., WALLIKER, D. 1991. cytochrome b in *Plasmodium falciparum*.
- DORE, E., PACE, T. 1991. repeats in *Plasmodium falciparum*.
- DYE, C.M. 1991. *Parasitology Today* 7: 236-237.
- FOLEY, M., RANFORD-CARTWRIGHT, R. 1991. for isolating malaria parasites. *Parasitology* 53: 241-244.
- FOOTE, S.J., THOMAS, J.C. 1991. multidrug resistance in *Plasmodium falciparum*.
- JANSE, C.J., BOORJAN, J., CASAGLIA, O., production, DNA sequencing, *in vivo*. *Experimental Parasitology* 68: 1-10.
- JANSE, C.J., RAMESH, R.J.M., Nucleic Acids Research 19: 1-10.
- PACE, T., PONZI, M., within telomeres of *Plasmodium falciparum* and *Cellular Biology*.
- POLOGE, L.P. and HANSEN, J. 1991. *falciparum* chromosome.
- RANFORD-CARTWRIGHT, R. 1991. hybrids of *Plasmodium falciparum*.
- RANFORD-CARTWRIGHT, R. 1991. cross-fertilization in *Plasmodium falciparum*.
- SCHERF, A. and MATHIAS, L. 1991. *Plasmodium falciparum* subtelomeric genes.
- SCHERF, A., CARTELO, J., PEREIRA da SILVA, A. 1991. by chromosome banding.
- SINDEN, R.E. and HANSEN, J. 1991. potential role in gametocyte development.
- SINNIS, P. and WEELE, C. 1991. *Journal of Protozoology* 38: 287-295.
- SNEWIN, V.A., HERGENROTHER, J. 1991. chromosomes: cross-fertilization.
- TANABE, K., MacKAY, D. 1991. 287-295.

- CORCORAN, L.M., THOMPSON, J.K., WALLIKER, D. and KEMP, D.J. 1988. Homologous recombination within sub-telomeric repeat sequences generates chromosome size polymorphisms in *Plasmodium falciparum*. *Cell* 53: 807-813.
- CREASEY, A., FENTON, B., WALKER, A., THAITHONG, S., OLIVEIRA, S., MUTAMBU, S. and WALLIKER, D. 1990. Genetic diversity of *Plasmodium falciparum* shows geographical variation. *American Journal of Tropical Medicine and Hygiene* 42: 403-413.
- CREASEY, A.M., RANFORD-CARTWRIGHT, L.C., MOORE, D.L., WILLIAMSON, D.H., WILSON, R.J.M., WALLIKER, D. and CARTER, R. 1993. Uniparental inheritance of the mitochondrial gene cytochrome b in *Plasmodium falciparum*. *Current Genetics*, in press.
- DORE, E., PACE, T., PONZI, M., PICCI, L. and FRONTALI, C. 1990. Organization of subtelomeric repeats in *Plasmodium berghei*. *Molecular and Cellular Biology* 10: 2423-2427.
- DYE, C.M. 1991. Population genetics of nonclonal, nonrandomly mating malaria parasites. *Parasitology Today* 7: 236-240.
- FOLEY, M., RANFORD-CARTWRIGHT, L.C. and BABIKER, H.A. 1992. Rapid and simple method for isolating malaria DNA from fingerprick samples of blood. *Molecular and Biochemical Parasitology* 53: 241-244.
- FOOTE, S.J., THOMPSON, J.K., COWMAN, A.F. and KEMP, D.J. 1989. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell* 57: 921-930.
- JANSE, C.J., BOORSMA, E.G., RAMESAR, J., van VIANEN, P., van der MEER, R., ZENOBI, P., CASAGLIA, O., MONS, B. and van der BERG, F.M. 1989. *Plasmodium berghei*: gametocyte production, DNA content and chromosome-size polymorphisms during asexual multiplication in vivo. *Experimental Parasitology* 68: 274-282.
- JANSE, C.J., RAMESAR, J. and MONS, B. 1992. Chromosome translocation in *Plasmodium berghei*. *Nucleic Acids Research* 20: 581-586.
- PACE, T., PONZI, M., DORE, E., JANSE, C., MONS, B. and FRONTALI, C. 1990. Long insertions within telomeres contribute to chromosome-size polymorphism in *Plasmodium berghei*. *Molecular and Cellular Biology* 10: 6759-6764.
- POLOGE, L.P. and RAVETCH, J.V. 1988. Large deletions result from breakage and healing of *P. falciparum* chromosomes. *Cell* 55: 869-874.
- RANFORD-CARTWRIGHT, L.C., BALFE, P., CARTER, R. and WALLIKER, D. 1991. Genetic hybrids of *Plasmodium falciparum* identified by amplification of genomic DNA from single oocysts. *Molecular and Biochemical Parasitology* 49: 239-244.
- RANFORD-CARTWRIGHT, L.C., BALFE, P., CARTER, R. and WALLIKER, D. 1993. Frequency of cross-fertilization in the human malaria parasite *Plasmodium falciparum*. *Parasitology*, in press.
- SCHERF, A. and MATTEI, D. 1992. Cloning and characterisation of chromosome breakpoints of *Plasmodium falciparum*: breakage and new telomere formation occurs frequently and randomly in subtelomeric genes. *Nucleic Acids Research* 20: 1491-1496.
- SCHERF, A., CARTER, R., PETERSEN, C., ALANO, P., NELSON, R., AIKAWA, M., MATTEI, D., PEREIRA da SILVA, L. and LEECH, J. 1992. Gene inactivation of Pf11.1 of *Plasmodium falciparum* by chromosome breakage and healing: identification of a gametocyte-specific protein with a potential role in gametogenesis. *EMBO Journal* 11: 2293-2301.
- SINDEN, R.E. and HARTLEY, R.H. 1985. Identification of the meiotic division of malarial parasites. *Journal of Protozoology* 32: 742-744.
- SINNIS, P. and WELLEMS, T.E. 1988. Long-range restriction maps of *Plasmodium falciparum* chromosomes: crossing-over and size variation among geographically distant isolates. *Genomics* 3: 287-295.
- SNEWIN, V.A., HERRERA, M., SANCHEZ, G., SCHERF, A., LANGSLEY, G. and HERRERA, S. 1991. Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in *Plasmodium falciparum* wild isolates from Colombia. *Molecular and Biochemical Parasitology* 49: 265-276.
- TANABE, K., MacKAY, M., GOMAN, M. and SCAIFE, J.G. 1987. Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *Journal of Molecular Biology* 195: 273-287.

- TIBAYRENC, M. and AYALA, F.J. 1991. Towards a population genetics of micro-organisms: the clonal theory of parasitic protozoa. *Parasitology Today* 7: 228–232.
- TIBAYRENC, M., KJELLBERG, F. and AYALA, F.J. 1990. A clonal theory of parasitic protozoa: the population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas* and *Trypanosoma* and their medical and taxonomical consequences. *Proceedings of the National Academy of Sciences of the USA* 87: 2414–2418.
- TRIGLIA, T. and KEMP, D.J. 1991. Large fragments of *Plasmodium falciparum* DNA can be stable when cloned in yeast artificial chromosomes. *Molecular and Biochemical Parasitology* 44: 207–212.
- TRIGLIA, T., WELLEMS, T.E. and KEMP, D.J. 1992. Towards a high resolution map of the *Plasmodium falciparum* genome. *Parasitology Today* 8: 225–229.
- WALKER-JONAH, A., DOLAN, S., GWADZ, R.W., PANTON, L.J. and WELLEMS, T.E. 1992. An RFLP map of the *Plasmodium falciparum* genome, recombination rates and favored linkage groups in a genetic cross. *Molecular and Biochemical Parasitology* 51: 313–320.
- WALLIKER, D. 1989. Implications of genetic exchange in the study of protozoan infections. *Parasitology* 99: S49–S58.
- WALLIKER, D. 1991. Malaria parasites: randomly interbreeding or 'clonal' populations? *Parasitology Today* 7: 232–235.
- WILSON, R.J.M., GARDNER, M.J., FEAGIN, J.E. and WILLIAMSON, D.H. 1991. Have malaria parasites three genomes? *Parasitology Today* 7: 134–136.

Genome maps in *Plasmodium* of the clonal

T.E. Wellems

Determinants of invasion and infectivity. Detailed knowledge of the parasite genome is essential to new targets for drug development. It is difficult to identify these targets by the relatively slow methods of contamination by receptors, enzyme assays, heterologous expression, and biochemical bases.

A non-biochemical approach to cloning, sometimes called linkage analysis, involves segments harbouring genes, then cloned as large fragments of the parasite genome. This is the agent of the mutation, the determinant of the tropical world.

MAPS OF THE *PLASMODIUM*

There are two types of map: the physical map and the physical map. The physical map, as measured by large numbers of progeny, is 1% recombination. The physical map is additive in the linkage analysis of individual chromosomes.

The physical map is based on measurements of genetic distance, nucleotide base pair distance, rates in linkage studies, and variable crossover rates.

Laboratory crosses are required, so physical maps are required.

D. Waller

GENOME ANALYSIS OF PROTOZOAN PARASITES

PROCEEDINGS OF A WORKSHOP HELD AT ILRAD
NAIROBI, KENYA
11-13 NOVEMBER 1992

Edited by
S.P. Morzaria

Leishmania brucei
stained gel was
ough ImagQuant

dom amplified
era. Illustration

THE INTERNATIONAL LABORATORY FOR RESEARCH ON ANIMAL DISEASES
BOX 30709 • NAIROBI • KENYA

ECLTM Highlights

Issue

5

Contents

Nucleic Acid Analysis

ECL probe-amp reagents

- Non radioactive labelling of DNA probes during PCR 2

- Ribotyping of *Staphylococcus aureus* using fluorescein labelled probes and ECL detection 4

Screening PCR products with oligonucleotides

- Use of ECL 3'-oligolabelling system for cystic fibrosis mutation screening 5

- Diversity of two antigen genes of malarial parasites detected by PCR and the ECL 3'-oligolabelling system 5

Mapping applications of the ECL Direct System

- The use of cycle reprobing for the construction of rice RFLP maps 7

- DNA fingerprinting of citrus cultivars 8

- The forensic application of ECL 9

Western Blot Analysis

- Protein molecular weight markers for ECL Western blotting 10

- Quantitation of pre-beta high density lipoprotein in plasma using immunoblotting and ECL detection 11

- Detection of protein tyrosine phosphorylation using ECL 13

- Use of ECL to study anti-endothelial antibodies and coronary artery disease after cardiac transplantation 15

- Product listing 16

- References 18

Screening PCR products with oligonucleotides

Use of ECL 3'-oligolabelling system for cystic fibrosis mutation screening.

A E Shrimpton and
D J H Brock, Human
Genetics Unit, Western
General Hospital, Edinburgh,
Scotland, EH4 2XU.

Human DNA samples can be screened for the presence of cystic fibrosis (CF) mutations within the cystic fibrosis transmembrane conductance regulator gene (CFTR) exon 9 using allele specific oligonucleotides (ASO). The ECL 3'-oligolabelling system can be used for probe labelling, hybridization and detection, as described below.

Methods

Genomic DNA samples are PCR amplified using primers specific to CFTR exon 9 (9i-3 and 9i-5)⁽⁸⁾ resulting in a 561 bp product, as determined by agarose gel analysis.

The PCR products are denatured and vacuum dot blotted onto Hybond-N+ membrane.

ASO's for the 5T, 7T and 9T⁽⁹⁾ variants in intron 8 and the A455E mutation in exon 9 are 3'-end labelled with fluorescein-11-dUTP using the ECL 3'-oligolabelling system (RPN 2130). Following hybridization to the dot blots and incubation with anti-fluorescein-horseradish peroxidase conjugate, the standard ECL protocol is followed for signal detection with a 2 minute exposure using Hyperfilm-ECL.

Results

Figure 5 shows 5T, 7T, 9T and A455E ASO hybridization results for seventeen individuals. Only the A455E positive control carries A455E. All samples can be scored unambiguously for their 5T, 7T and 9T genotype at the intron 8-exon 9 boundary.

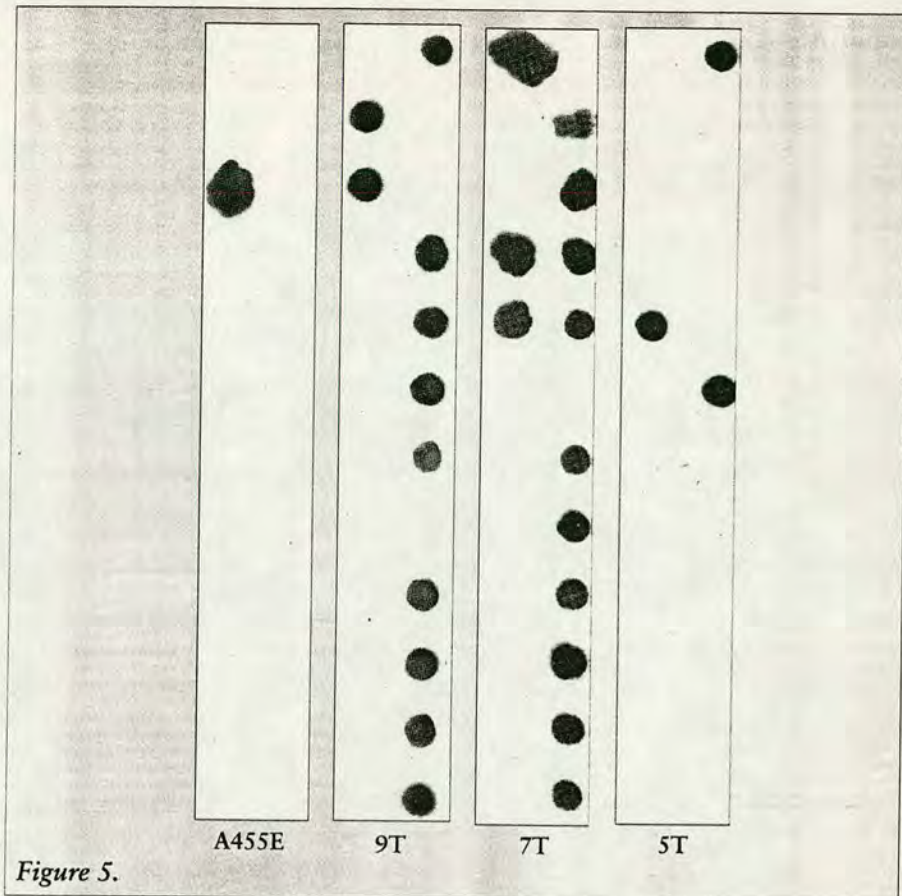


Figure 5.

Diversity of two antigen genes of malaria parasites detected by PCR and the ECL 3'-oligolabelling system.

Hamza Babiker and Lisa Ranford-Cartwright
Institute of Cell, Animal and Population Biology,
University of Edinburgh, Scotland.

Introduction

Malaria is a major health problem in many tropical countries. In Africa alone, at least 300-500 million malaria episodes are treated annually and up to 1 million deaths among children are attributed to the disease⁽¹⁰⁾. The parasite infects the human host in a haploid form which multiplies asexually. There is a considerable interest in developing a vaccine against the most pathogenic species of the parasite, *Plasmodium falciparum*. The objective of this work was to investigate the polymorphism of two antigen genes, MSP-1 and MSP-2, in the parasites found in a small village in Tanzania; these antigens have been proposed as candidates for a vaccine.

Methods

DNA was extracted from *P. falciparum* isolates, collected from infected individuals in the village, by the method of Foley *et*

al⁽¹¹⁾. Each DNA sample was then amplified by the polymerase chain reaction (PCR)⁽¹²⁾ using primers recognising conserved regions of the MSP-1 and MSP-2 genes^(13,14). These regions are on either side of areas of tandem amino acid repeats which vary both in number and sequence in different alleles of each antigen gene (see figure 6). There are three known sequence variants of the repetitive region of the MSP-1 gene, denoted the K1, MAD20 and RO33, and two sequence variants of the MSP-2 gene repetitive region, denoted the IC1 and FC27 types.

To investigate the distribution of the different alleles of these two genes in the parasite population, amplified PCR fragments were Southern blotted to nylon membranes (Genescreen)⁽¹⁵⁾. Each of the three allelic types of MSP-1 and the two types of MSP-2 could be distinguished by probing the

Southern blots with allele-specific oligonucleotides^(14,16).

These oligonucleotides were first labelled using the Amersham ECL 3'-oligolabelling kit (RPN 2130). 100 x 10⁻¹² moles of each oligonucleotide were labelled with fluorescein-11-dUTP according to the manufacturer's protocol. Briefly, the oligonucleotide and the fluorescein-dUTP were incubated with terminal transferase for 60-90 minutes at 37°C. The labelled oligonucleotides could be kept at -20°C for more than two months and the same batch gave reproducible results after this period.

Hybridization of the labelled

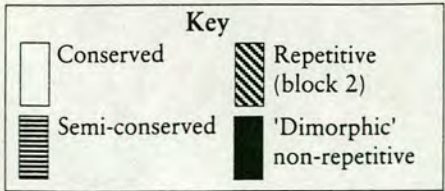
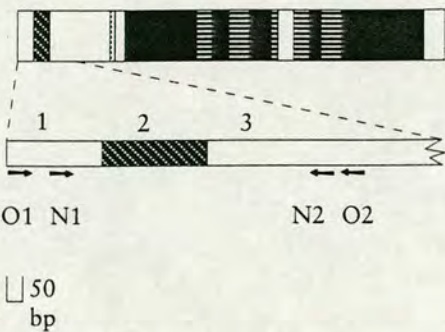
oligonucleotides with the Southern blots was carried out for one hour, with a probe concentration of 5-10ng/ml. The blots were first washed with 1 x SSC at room temperature, followed by higher stringency washes with 0.1 x SSC for 30 minutes at a temperature 5°C below the T_m of each oligonucleotide. Hybridized oligonucleotide was detected using the reagents provided in the kit. Amersham Hyperfilm was used for signal detection. Each blot was exposed to the film for 1-2 minutes and the film was then developed.

Results

Each PCR fragment was found to hybridize to at least one labelled oligonucleotide (see figure 7). Some

PCR fragments hybridized to more than one allele-specific probe, indicating that there are either two alleles of the same size but with a different sequence or a hybrid allele. This technique is very suitable for use in developing countries where radioactive labelling may not be feasible, and where disposal of radioactivity is difficult and hazardous. We have found that ECL-labelled oligonucleotides can be kept in the freezer for much longer than ³²P-labelled oligonucleotides. The combination of the PCR technique and the ECL-system provides a powerful tool for population genetic analysis of variant genes, such as the malaria parasite antigens.

MSP 1



MSP 2

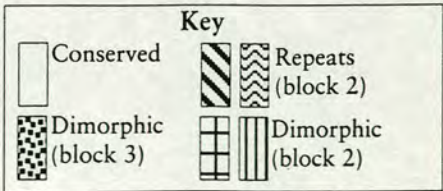
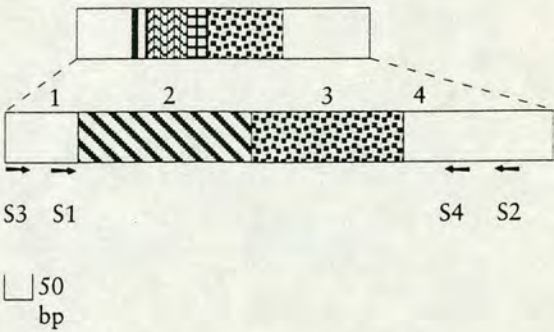


Figure 6. Diagrammatic representation of the merozoite surface protein genes, MSP-1 and MSP-2, of *Plasmodium falciparum*. The regions amplified are shown enlarged with the positions of the primers marked with arrows. The conditions used for the polymerase chain reaction and the sequences of the primers are as in reference 11.

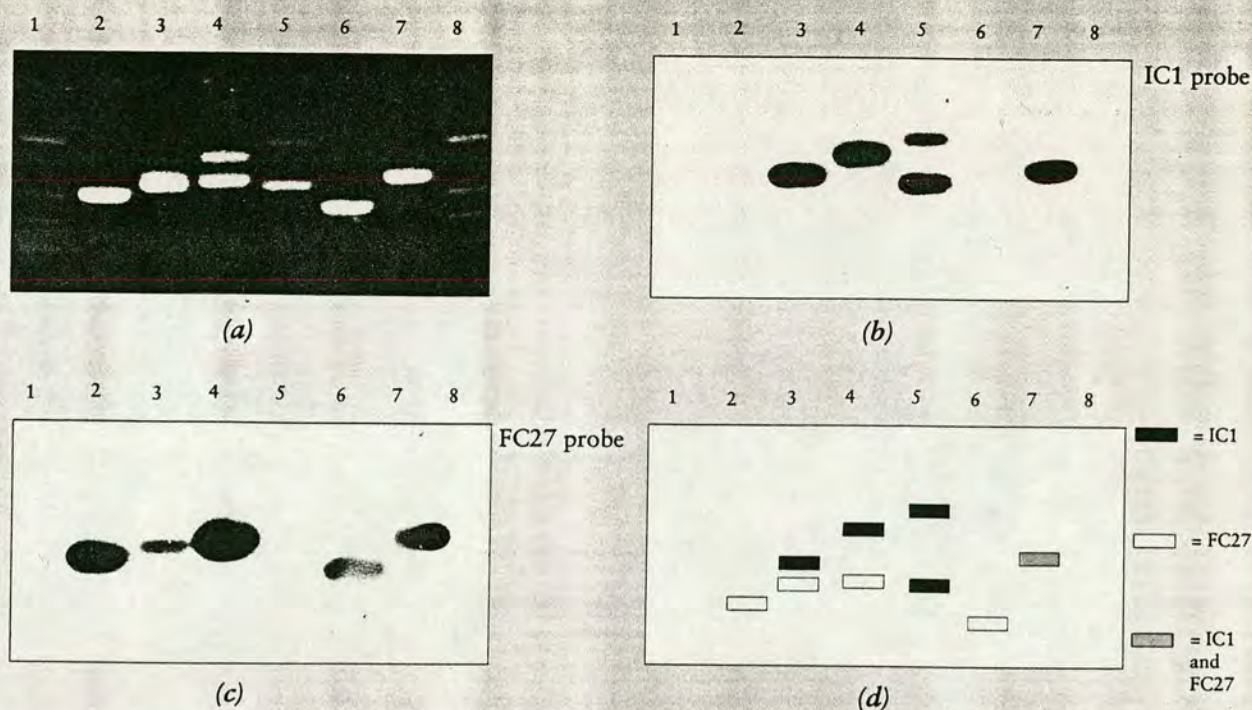


Figure 7. PCR-amplified fragments of the MSP-2 gene of *P. falciparum* collected from a Tanzanian village. (a) shows the fragments separated on a 1.6% agarose gel stained with ethidium bromide. The fragments were transferred to nylon and the Southern blot was hybridized with two allele-specific oligonucleotides specific for the MSP-2 dimorphic block 3, as shown in figure 6, which were labelled using the ECL-3' oligolabelling kit, (b and c) show the autoradiographs from these hybridizations. The sequence of the probes is given in reference 17 (d) shows a schematic representation of the results.

Lanes 2 and 6 each contain a single fragment hybridizing to the FC27 oligonucleotide only. Lanes 3 and 4 each contain two bands, one of which hybridizes to the FC27 oligonucleotide and the other to the IC1 oligonucleotide. Lane 5 contains two bands, both of which hybridize to the IC1 oligonucleotide. Lane 7 contains a single band which hybridizes to both of the oligonucleotide probes, and is therefore either a hybrid allele, or two alleles of the same size but different sequence. Lanes 1 and 8 are size markers.

Mapping applications of the ECL direct system

The use of cycle reprobating for the construction of rice RFLP maps using the ECL direct system.

Nori Kurata and
Yuuzou Minobe,
The Rice Genome
Research Program,
Japan

Introduction

For the construction of detailed RFLP maps of plant genomic DNA, numerous markers should be analyzed on the parent DNAs and on the large number of DNAs from crossed F_2 progenies. A key point of such experiments is efficient detection of Southern blots of the

plant DNA. We have been successfully applying the ECL direct nucleic acid labelling and detection system (Amersham) to rice RFLP mapping. We reprobe each blot over 20 times using a rapid and efficient protocol.

Experimental details

Target DNA: target DNA was prepared from 190 F_2 rice plants and digested with one of 8 restriction enzymes. 2 μ g aliquots were electrophoresed on gels containing 190 lanes. The DNA was blotted onto positively charged nylon membrane and fixed by baking.

Probes: Probe DNAs of various rice cDNA clones, YAC clones, Not I-linking clones and randomly selected genomic clones were amplified by PCR and labelled with horseradish peroxidase using the ECL direct

nucleic acid labelling and detection system.

Hybridization, detection and reprobating: Seven membranes (150cm² each) were hybridized in 50ml of the ECL gold hybridization buffer supplied with the system, together with 10-20ng/ml labelled probe at 42°C overnight. Following washing and incubation with ECL detection reagents, the chemiluminescence was detected on X-ray film using a 2-hour exposure. For reprobating experiments, the filter was washed and rehybridized without stripping the membrane, according to the procedures given with the ECL system.

Results and conclusions

We could detect clear hybridization signals after reprobating more than 25 times, as shown in figure 8. The

References

- 1) CUNNINGHAM, M. W., *Life Science* (An Amersham International plc publication), 6, 1991.
- 2) WHITEHEAD, T. P. *et al.*, *Nature*, 305, pp.158-159, 1983.
- 3) SAIKI, R. K. *et al.*, *Science*, 230, pp.1350-1354, 1985.
- 4) SAIKI, R. K. *et al.*, *Science*, 239, pp.487-491, 1985.
- 5) RIGBY, P. W. J. *et al.*, *J. Mol. Biol.*, 113, pp.237-251, 1977.
- 6) FEINBERG, A. P. and B. A. VOGELSTEIN, *Anal. Biochem*, 132, pp.6-13, 1983.
- 7) GUSTAFERRO, C. A. and PERSING, D. H., *J. Clinical Micro*, 30, pp.1039-1041, 1992.
- 8) KEREM, B.S. *et al.*, *PNAS*, pp.8447-8451, 1990.
- 9) CHU, C.S. *et al.*, *EMBO*, pp.1355-1363, 1991.
- 10) DEMING, *et al.*, *Bull WHO*, 67, pp.695-700, 1989.
- 11) FOLEY, *et al.*, *Mol. Biochem. Parasitol*, 53, pp.241-244, 1992.
- 12) SAIKI, *et al.*, *Science*, 239, pp.487-491, 1988.
- 13) RANFORD-CARTWRIGHT, *et al.*, *Mol. Biochem. Parasitol*, 49, pp.239-244, 1991.
- 14) SNEWIN, *et al.*, *Mol. Biochem. Parasitol*, 49, pp.265-276, 1991.
- 15) SOUTHERN, E. M., *J. Mol. Biol.*, 98, pp.503-517, 1975.
- 16) PETERSON, *et al.*, *Mol. Cell. Biol.*, 8, pp.2664-2667, 1988.
- 17) BABIKER, *et al.*, in preparation.
- 18) JEFFREYS, A. J. *et al.*, *Nature*, 314, pp.67-73, 1985.
- 19) JEFFREYS, A. J. *et al.*, *Nature*, 316, pp.76-79, 1985.
- 20) LAEMMLI, U. K., *Nature*, 227, pp.681, 1970.
- 21) TOWBIN, H., *PNAS*, 76, pp.4350, 1979.
- 22) HOFFMAN, W. L. and JUMP, A. A., *Anal. Biochem.*, 181, pp.318, 1989.
- 23) NEARY, R. *et al.*, *Atherosclerosis*, 89, pp.35-48, 1991.
- 24) O'KANE, M. J. *et al.*, *Clinical Chemistry* (in press).
- 25) ROSS, A. H. *et al.*, *Nature*, 294, pp.654-656, 1981.
- 26) FRACKELTON, A. R. *et al.*, *Methods Enzym.*, 201, pp.79-92, 1991.
- 27) COOPER, J. A. and HUNTER, T., *Curr. Top. Microbiol. Immunol.*, 107, pp.126-159, 1983.
- 28) FABRICANT, R. N. *et al.*, *PNAS*, 74, pp.565-569, 1977.
- 29) GREENE, L. A. and TISCHLER, A. S., *PNAS*, 73, pp.2424-2428, 1976.
- 30) BANNER, N. R. *et al.*, *Clinical Transplants*, pp.17-26, 1987, ed. Terasaki, P., Los Angeles: UCLA Tissue Typing Laboratory.
- 31) GAO, S. *et al.*, *Seminars in Thoracic and Cardiovascular Surgery*, pp.241-49, 1990, eds. Yacoub, M. H., Loop FD, Philadelphia: Saunders.
- 32) ROSS, R., *New Engl. J. Med.*, 314, pp.488-500, 1986.
- 33) JAFFE, E. A. *et al.*, *J. Clin. Invest.*, 52, pp.2745-56, 1973.
- 34) BRADFORD, B., *Anal. Biochem.*, 72, pp.248-54, 1976.
- 35) SIMMONDS, J. *et al.*, in, 'Proceedings of the international meeting on two-dimensional electrophoresis', London, National Heart and Lung Institute, pp.46-48, 1991, ed. Dunn, M.J.
- 36) DUNN, M. J. *et al.*, *Transplantation*, 51, pp.806-12, 1991.

Genetic evidence that RI chloroquine resistance of *Plasmodium falciparum* is caused by recrudescence of resistant parasites

Hamza Babiker^{1,2}, Lisa Ranford-Cartwright¹, Ali Sultan^{1,3}, Gwiria Satti³ and David Walliker¹ ¹Institute of Cell, Animal and Population Biology, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JN, Scotland, UK; ²Department of Malaria Administration, Ministry of Health, Khartoum, Sudan; ³Department of Biochemistry, Faculty of Medicine, University of Khartoum, Sudan

Abstract

Isolates of *Plasmodium falciparum* from patients in a Sudanese village exhibiting RI resistance to chloroquine have been typed for allelic variants of 2 merozoite surface antigens, MSP1 and MSP2. Blood forms were taken from each patient before chloroquine was administered, and after parasites had reappeared following treatment. Each patient was found to be infected with genetically different parasites. However, in each patient the parasites of the recrudescence infections possessed the same alleles of each gene as those of the primary infection. The results show that the parasites which reappeared after chloroquine were a genuine recrudescence of the primary forms, and not derived from a new infection.

Introduction

Chloroquine resistance in *Plasmodium falciparum* is conventionally defined as RI (low), RII (intermediate) or RIII (high), depending on the clinical response of patients to a standard course of treatment with the drug (BRUCE-CHWATT, 1986). In RI resistance, parasites are temporarily cleared from the blood following chloroquine treatment, but reappear several days after the treatment is terminated. These parasites are usually considered to represent a recrudescence of the initial parasites which are resistant. Alternatively, they could be due to reinfection of the patient with fresh parasites. Such parasites might exhibit a different drug response from those of the primary infection, since it is well known that mixtures of resistant and sensitive parasites occur within a single region (see, e.g., BABIKER *et al.*, 1991b).

During surveys of *P. falciparum* among inhabitants of a village in eastern Sudan in 1989 and 1990, it was found that many isolates exhibited resistance *in vitro* to chloroquine (BABIKER *et al.*, 1991b). Some patients who had been treated with chloroquine following diagnosis were found to exhibit parasites as well as febrile symptoms after periods of one to 4 weeks following treatment. In the study reported here, we typed parasites of the recrudescence and primary infections of these patients for 2 highly polymorphic antigen genes, using the polymerase chain reaction (PCR) combined with the use of non-radiolabelled chemiluminescent gene probes. We showed that, in each patient, the recrudescence parasites possessed the same alleles of these genes as the primary parasites. The work thus showed unequivocally that the secondary parasites were a genuine recrudescence, and not the products of a new infection.

Subjects, Materials and Methods

Study area and patients

Details of the study area, Asar Village in eastern Sudan, have been given previously (BABIKER *et al.*, 1991a, 1991b). In this area, malaria transmission occurs from September to November, following seasonal rains. The principal parasite is *P. falciparum*, causing more than 90% of the malaria cases (EL-GADDAL, 1986). Drug tests *in vitro* have shown that the parasite population of Asar contains a diversity of chloroquine-resistant and chloroquine-sensitive forms (BABIKER *et al.*, 1991b).

Blood films were prepared from patients attending a daily clinic in Asar throughout the transmission seasons of 1989 and 1990. On diagnosis of *P. falciparum*, venous blood samples were immediately taken from each patient, with their consent, and cryopreserved in liquid nitrogen (BABIKER *et al.*, 1991a). Each patient was then treated with a standard dose of chloroquine of 25 mg/kg over three 3 d and examined daily for clearance of parasitaemia, which occurred by day 7 or earlier if the parasites were sensitive to the drug. Six patients returned to the clinic with febrile symptoms after periods ranging from

10 to 30 d, all of whom proved to have *P. falciparum* in their blood. A further sample of blood was taken and cryopreserved. The patients were then treated with Fansidar[®] (sulfadoxine/pyrimethamine), to take account of the possibility that the parasites could be resistant to chloroquine.

Tests *in vitro* for chloroquine resistance

The isolates which caused the primary infections in the 6 patients who later returned to the clinic were established in culture *in vitro* and tested for chloroquine response, using graded doses of the drug in microtitre plates (BABIKER *et al.*, 1991b). The minimum inhibitory concentration (MIC) which killed all, or nearly all, the parasites was estimated by microscopical examination of Giemsa-stained blood smears.

Genotyping parasites

Parasites were characterized for alleles of genes determining 2 polymorphic merozoite surface proteins, denoted MSP1 and MSP2. Both these genes contain regions encoding tandemly repeating amino acids, which may vary in both number and sequence in different alleles (TANABE *et al.*, 1987; SMYTHE *et al.*, 1991). Variations in the length of these regions can be readily identified as size differences on agarose gels of fragments amplified by PCR, using primers recognizing conserved sequences flanking the repeats (FOLEY *et al.*, 1992; RANFORD-CARTWRIGHT *et al.*, 1993). The sequence variations in this region of MSP1 can be grouped into 3 types, denoted K1, MAD20 and Ro33 after the isolates from which they were originally described (KIMURA *et al.*, 1990). For MSP2, 2 types of sequence have been found, denoted IC1 and FC27 (SNEWIN *et al.*, 1991).

PCR amplification of MSP1 and MSP2 genes. *P. falciparum* deoxyribonucleic acid was first isolated from each sample of parasitized blood using the method of FOLEY *et al.* (1992). PCR was used to amplify the repeat region of MSP1, denoted block 2 by TANABE *et al.* (1987), and the central region of MSP2 which includes repeat sequences (FENTON *et al.*, 1991; SMYTHE *et al.*, 1991). Details of the primers used, the PCR conditions, and electrophoresis of the amplified products have been given by RANFORD-CARTWRIGHT *et al.* (1993). Southern blots of the gels were made on to nylon membranes (SAMBROOK *et al.*, 1989).

Probing of PCR-amplified products. Oligonucleotide probes were made which recognized K1-, MAD20- and Ro33-type sequences of MSP1, and IC1- and FC27-type sequences of MSP2. The sequences of the MSP1 probes have been given by SNEWIN *et al.* (1991). The sequences of the MSP2 probes were IC1: 5'-GCAGAAGCATC-TACCAGTACC-3' and FC27: 5'-CACCTTCACCACC-CATCAC-3'. The oligonucleotides were labelled using the Amersham Enhanced Chemiluminescence[®] (ECL) 3' oligolabelling kit. 1×10^{-10} moles of each oligonucleotide

were labelled with fluorescein-11-d-uridine triphosphate by incubation with terminal transferase for 60–90 min at 37°C, according to the manufacturer's instructions.

Hybridization of the labelled oligonucleotides with Southern blots was carried out according to the manufacturer's instructions, with the following modifications. Hybridization was for 1 h at temperatures of 56°C, 68°C and 74°C for MSP1 probes MAD20, Ro33 and K1 respectively, and 56°C and 59°C for MSP2 probes FC27 and IC1 respectively. The probes were used at concentrations of 5–10 ng/mL of hybridization solution. The blots were first washed with standard saline citrate (1× SSC) 0.1% sodium dodecyl sulphate (SDS) at room temperature, followed by higher stringency washes with 0.1× SSC/0.1% SDS for 30 min at the same hybridization temperatures. Hybridized oligonucleotides were detected using the Amersham ECL detection kit. Each blot was exposed wet, wrapped in Saranwrap[®], to ECL-Hyperfilm[®] (Amersham) for 1–2 min before being developed. Following each hybridization, the probes were stripped from the membranes by boiling in 0.1× SSC/1% SDS for 15 min, followed by rinsing in 5× SSC for 5 min with continuous agitation (SAMBROOK *et al.*, 1989), and stored in Saranwrap[®] at -20°C for eventual hybridization with other probes.

Table 1. Chloroquine responses *in vitro* of *P. falciparum* parasites from initial infections from six patients, taken before commencement of drug treatment

Parasites	MIC ^a (× 10 ⁻⁶ M)
Patient isolates	
107/89	0.4
123/89	0.8
101/90	0.8
103/90	1.6
120/90	1.6
124/90	1.6
Control clones	
3D7 ^b	0.2
Dd2 ^c	1.6

^aMinimum inhibitory concentration (which kills all, or nearly all, parasites).

^bChloroquine sensitive.

^cChloroquine resistant.

Table 2. Alleles of MSP1 and MSP2 genes of *P. falciparum* from primary and recrudescence (R) infections in patients treated with chloroquine

Patient isolate no.	Age (years)	Date sampled	MSP1 alleles		MSP2 alleles	
			Size ^a	Sequence ^b	Size ^a	Sequence ^b
107/89	30	25.10.89	470	Ro33	650	IC1
107/89-R		24.11.89	470	Ro33	650	IC1
123/89	17	31.10.89	540	MAD20	540	IC1
123/89-R		23.11.89	470	Ro33	540	IC1
101/90	8	7.10.90	530	K1	580	FC27
101/90-R		26.10.90	530	K1	580	FC27
103/90	15	20.10.90	470	Ro33	540	FC27
103/90-R		9.11.90	470	Ro33	540	FC27
120/90	50	29.10.90	470	K1	n.d.	n.d.
120/90-R		13.11.90	470	K1	580	FC27
124/90	20	1.11.90	470	Ro33	n.d.	n.d.
124/90-R		10.11.90	470	Ro33	540	IC1

^aEstimated number of base pairs of DNA fragments of each allele amplified by the polymerase chain reaction on agarose gels; n.d.=not determined.

^bThe probe which hybridised with each amplified fragment; n.d.=not determined.

Results

Tests for chloroquine response

Tests in vivo. All the 6 patients who exhibited recrudescences showed an RI type of chloroquine resistance response. In 2 patients, parasitaemias were cleared, but reappeared within 7 d. The other 4 patients showed late recrudescences 2–3 weeks after completion of treatment.

Tests in vitro. The chloroquine response of parasites from the primary infections of each of the 6 patients was tested *in vitro* (Table 1). The MICs ranged from 0.4 × 10⁻⁶M to 1.6 × 10⁻⁶M, compared to 0.2 × 10⁻⁶M for the drug-sensitive control clone 3D7.

MSP1 and MSP2 characterization of parasites

PCR followed by hybridization of blots with non-radio-labelled probes was used to identify MSP1 and MSP2 alleles of the parasites from the 6 patients who had recrudescences of *P. falciparum* following chloroquine treatment, as well as from 64 further patients in the village.

Parasites from chloroquine-treated patients. The MSP1 and MSP2 alleles of the parasites from these patients are shown in Table 2. The Figure illustrates the MSP2 alleles of primary and recrudescence isolates from 3 of these patients. Each patient was found to contain parasites with different combinations of alleles of the 2 genes. The recrudescence parasites in each patient possessed alleles identical to those of the parasites of the primary infections.

In one patient (no. 123/89), two alleles of MSP1 were seen in both primary and recrudescence samples, showing that this infection contained a mixture of at least 2 genetically distinct chloroquine-resistant parasite clones. In the other 5 patients, only single alleles of each gene were detectable, suggesting clonal-type infections in each case; however, it cannot be entirely excluded that these patients contained mixtures of parasites which by chance were identical at the MSP1 and MSP2 loci, but differed at other loci not examined in this work.

Parasites of other patients in the village. Seventy patients from Asar, including 6 showing recrudescence infections, were examined for MSP1 and MSP2 alleles. Eleven alleles of MSP1 were found among these isolates, characterized by PCR products ranging in length from 470 to 580 base pairs (bp), as well as by K1-, MAD20-, or Ro33-type sequences. Sixteen alleles of MSP2 were found, with PCR products ranging in size from 470 to

700 bp and containing either IC1 or FC27 sequences. Only 2 of the 70 patients examined contained parasites with an identical combination of MSP1 and MSP2 alleles; these isolates were from 2 children (sisters) from

^aSSC was prepared at 20× normal strength as sodium chloride 3M and trisodium citrate 0.3M, pH 7.0, and then diluted to 5×, 1× and 0.1× as required.

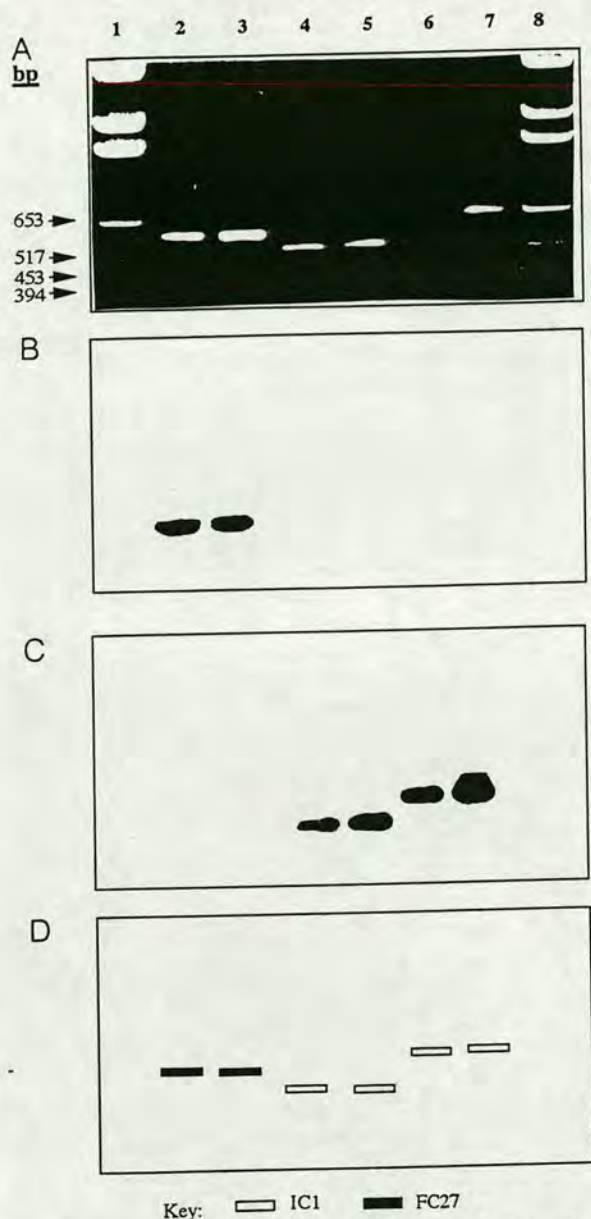


Figure. *MSP2* alleles in isolates from 3 patients with *P. falciparum* malaria, classified by size and sequence. Primary and recrudescent (R) isolates from each patient were run side by side. Tracks 1 and 8, size markers; 2, 101/90; 3, 101/90-R; 4, 123/89; 5, 123/89-R; 6, 107/89; 7, 107/89-R. A. Polymerase chain reaction fragments separated on 1.6% agarose gel and stained with ethidium bromide (bp=base pairs); the fragments were blotted on to a nylon membrane and hybridized separately with the allele-specific IC1 and FC27 probes. B. Film exposed to blot hybridized with FC27 probe. C. Film exposed to blot hybridized with IC1 probe. D. Schematic representation of the results.

the same household, who presented at the clinic on the same day.

Discussion

This work has demonstrated that parasites reappearing in the blood of patients suspected of having chloroquine resistant (RI) *P. falciparum* possess the same alleles of *MSP1* and *MSP2* as the parasites of the corresponding primary infections. Tests *in vitro* have confirmed that the primary parasites were slightly or moderately chloroquine resistant when compared to a control drug sensitive clone. We conclude that the recrudescent forms were derived directly from those of the resistant primary parasites, and not from a reinfection with new parasites.

Extensive polymorphisms of the *MSP1* and *MSP2* genes occur among the parasites of Asar village. In our previous study, 8 alleles of *MSP1* and 4 of *MSP2* were detected in the 1989 samples (BABIKER *et al.*, 1991a), using a panel of monoclonal antibodies specific for each antigen. In the current work, 11 alleles of *MSP1* and 16 of *MSP2* have been identified by the PCR/hybridization technique in samples collected in the 2 years 1989 and 1990. Most of the epitopes recognized by the monoclonal antibodies were not in the repeat regions of the antigens, and so no clear correlation could be made between the polymorphisms revealed by each technique. However, the combined results of the 2 studies show that the number of allelic variants of these antigens in the parasites of Asar is very high.

The existence of 11 alleles of *MSP1* and 16 of *MSP2* means that 176 (11×16) possible combinations of the alleles of each gene could occur among the parasites in the village. Although some alleles of each gene are more frequent than others (unpublished observations), the probability that 2 parasite clones taken at random from the inhabitants will have identical alleles at these loci is very low. Among the 70 isolates examined, only 2 had the same allele combinations, and these were from 2 sisters sleeping in the same house who had most probably been infected by the same mosquito on the same day. The probability that a single person will become reinfected from mosquitoes with parasites with *MSP1* and *MSP2* alleles identical to those of the primary infection is also exceedingly low. For these reasons, we conclude that the parasites reappearing in the blood of the 6 chloroquine-treated patients discussed in this paper were a genuine recrudescence of the primary parasites, and not a reinfection.

It was also of interest that the parasites in the 6 patients caused febrile episodes on each occasion they presented at the clinic. The causes of a new episode of malaria illness in a single patient are not understood, although one possible explanation is infection with a novel 'strain' of *P. falciparum*, to which the patient has not been previously exposed. (LINES & ARMSTRONG, 1992). It seems clear that this was not the case with the patients examined in the present study.

The method described here of genotyping *P. falciparum* for *MSP1* and *MSP2* alleles is similar to some of those previously described (e.g., by KIMURA *et al.*, 1990 and MERCEREAU-PUJALON *et al.*, 1991), except that we have made use of fluorescein-labelled probes in the hybridization work. As discussed elsewhere (e.g., by FOLEY *et al.*, 1992), the PCR technique is particularly sensitive and useful for this type of work, since it can be carried out with low numbers of parasites without the necessity of culturing them. Fluorescein-labelled probes, as used here, have obvious advantages over radio-labelled material. They are easy to make, and probably cheaper in the long term because they can be stored for long periods without loss of activity. They are particularly suitable for use in countries where radioactivity is difficult to obtain, and they have none of the hazards associated with the handling and disposing of radioactivity. The method is thus of great potential value in field surveys of genes in populations of parasites such as *Plasmodium*.

The prevalence of chloroquine resistance in Asar was found to be high in 1989 (BABIKER *et al.*, 1991b), resistant *P. falciparum* having been detected in this area since 1986 (BAYOUMI *et al.*, 1989). RII and RIII types of chloroquine resistance are easy to diagnose and alternative antimalarial drugs can be prescribed immediately. However, symptoms caused by recrudescent RI parasitaemias may frequently be confused by clinicians with other conditions, for which quite different treatments may be described. Thus, patients infected with RI chloroquine-resistant *P. falciparum* may harbour their parasites for a long time, with obvious epidemiological significance, especially if they have gametocytaemia. Correct diagnosis of recrudescent RI parasites is therefore important, and

has a practical impact on management of *P. falciparum* malaria in areas where resistance occurs. The techniques described here for characterizing parasites are at present expensive and need well-trained personnel; however, PCR technology is already finding a place in the diagnosis of malaria (see, e.g., JAUREGUIBERRY *et al.*, 1990), and we envisage that the methods used here might eventually be developed for field use.

Acknowledgements

Funding for this work has come from the UK Medical Research Council, the World Health Organization and the USA National Institutes of Health grant AI-16312. Hamza Babiker and Ali Sultan are recipients of British Council studentships. We appreciate particularly the support of the Malaria Administration of the Sudanese Ministry of Health, together with the Malaria Committee in Gedaref. We gratefully acknowledge the assistance of Lars Hviid and Thor Theander, supported by the Danish International Development Agency (DANIDA), as well as the great help of Riad Bayoumi and James Jensen. We thank especially the people of Asar village for their kind assistance and goodwill in this project.

References

Babiker, H. A., Creasey, A. M., Fenton, B., Bayoumi, R. A. L., Arnot, D. E. & Walliker, D. (1991a). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85, 572-577.

Babiker, H. A., Creasey, A. M., Bayoumi, R. A. L., Walliker, D. & Arnot, D. E. (1991b). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the *mdr1* genotype of recent isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85, 578-583.

Bayoumi, R. A., Babiker, H. A., Ibrahim, S. M., Galib, H. W., Saeed, B. O., Khider, S. & Karim, E. A. (1989). Spread of chloroquine resistant *Plasmodium falciparum* in Sudan. *Acta Tropica*, 46, 157-165.

Bruce-Chwatt, L. J., editor. (1986). *Chemotherapy of Malaria*, 2nd edition. Geneva: World Health Organization.

El Gaddal, A. (1986). Malaria in the Sudan. In: *Proceedings of the Conference on Malaria in Africa*, Buck, A. A. (editor). Washington: American Institute of Biological Sciences, pp. 156-159.

Fenton, B., Clark, J. T., Anjam Khan, C. M., Robinson, J. V., Walliker, D., Ridley, R., Scaife, J. G. & McBride, J. S.

(1991). Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen (MSA-2) of the malaria parasite *Plasmodium falciparum*. *Molecular and Cellular Biology*, 11, 963-971.

Foley, M., Ranford-Cartwright, L. C. & Babiker, H. A. (1992). Rapid and simple method for isolating material DNA from fingerprick samples of blood. *Molecular and Biochemical Parasitology*, 53, 241-244.

Jaureguiberry, G., Hatin, I., d'Auriol, L. & Galibert, G. (1990). PCR detection of *Plasmodium falciparum* by oligonucleotide probes. *Molecular and Cellular Probes*, 4, 409-414.

Kimura, E., Mattei, D., Di Santi, S. M. & Scherf, A. (1990). Genetic diversity in the major merozoite surface antigen of *Plasmodium falciparum*: high prevalence of a third polymorphic form detected in strains derived from malaria patients. *Gene*, 91, 57-62.

Lines, J. & Armstrong, J. R. M. (1992). For a few parasites more: inoculum size, vector control and strain-specific immunity to malaria. *Parasitology Today*, 8, 381-383.

Mercereau-Puijalon, O., Fandeur, T., Bonnefoy, S., Jacquemot, C. & Sarthou, J.-L. (1991). A study of the genomic diversity of *Plasmodium falciparum* in Senegal. 2. Typing by the use of the polymerase chain reaction. *Acta Tropica*, 49, 293-304.

Ranford-Cartwright, L. C., Balfe, P., Carter, R. & Walliker, D. (1993). Frequency of cross-fertilization in the human malaria parasite *Plasmodium falciparum*. *Parasitology*, 107, 11-19.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Smythe, J. A., Coppel, R. L., Day, K. P., Martin, R. K., Oduola, A. M. J., Kemp, D. J. & Anders, R. F. (1991). Structural diversity in the *Plasmodium falciparum* merozoite surface antigen MSA-2. *Proceedings of the National Academy of Sciences of the USA*, 88, 1751-1755.

Snewin, V. A., Herrera, M., Sanchez, G., Scherf, A., Langsley, G. & Herrera, S. (1991). Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in *Plasmodium falciparum* wild isolates from Colombia. *Molecular and Biochemical Parasitology*, 49, 265-276.

Tanabe, K., Mackay, M., Goman, M. & Scaife, J. G. (1987). Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *Journal of Molecular Biology*, 195, 273-287.

Received 12 August 1993; revised 13 September 1993; accepted for publication 14 September 1993

ALL THORS PROOF
PLEASE RETURN
Signed
Date